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Interspecific Homology between Ribosomal Proteins: The Characterization of a 30 S Ribosomal Protein from Bacillus Stearothermophilus Which Functionally Replaces Escherichia Coli S 19

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INTERSPECIFIC HOMOLOGY BETWEEN RIBOSOMAL PROTEINS: The Characterization of a 30 S Ribosomal Protein from *Bacillus stearothermophilus* which Functionally Replaces *Escherichia coli* S 19.

by

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For my Mother and Father . . . and Michael

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INTRODUCTION

Elucidation of the 3 dimensional structure of the ribosome and the assignment of a functional role to its RNA and protein components is one of the major goals of molecular biology. While the major steps in protein synthesis are now known in some detail, the interactions and functions of the various ribosomal components are just becoming understood.

It was not until the 1950's (21) that the ribonucleoprotein "microsomal fraction" of the rat liver cell was shown to be an absolute requirement for protein synthesis. The significance of the RNA and proteins was not appreciated at that time but the promulgation of the "adapter" hypothesis (7, 16), and the "messenger tape" theory (37, 49) in the late 1950's, naturally led to the investigations of the nature of the RNA and protein in microsomes or ribosomes.

It was known that protein synthesis was associated with RNA synthesis (2, 3, 4) but it was incorrectly hypothesized that for each protein, or perhaps for every polypeptide chain, one RNA molecule was assembled on the chromosomal DNA and searched its way out into the cytoplasm to form a stable, metabolically active particle (the ribosome) on which one species of protein was synthesized. In 1958, however, Pardee, Jacob and Monod (35) produced heteromerozygotes for the lac operon in conjugating *Escherichia coli*.

They found that when the recipient lac^- mutant received the wild type gene, synthesis of inducible protein started within three or four minutes. The synthesis of protein did not build up gradually, as one would expect if the RNA in the ribosomes acting as β-galactosidase templates had to be synthesized *de novo*. Thus it was concluded that ribosomal RNA was not acting as a template for each protein but perhaps performed some structural role.

The next problem was to determine what fraction of the total protein of the ribosomes consisted of growing or newly completed polypeptide chains, and what the chemical nature of the remainder of the ribosomal protein e.g. the structural component was. Calculations of bound amino acids in *in vitro* experiments with *E. coli* (8, 42), indicated that the growing peptide chain represented less than 5 per cent of the total ribosomal protein. In 1961 Waller and Harris (48) showed by NH_2-terminal analysis that ribosomal protein was not a random sample of cellular protein but a class of basic proteins which possibly served to maintain the ribosomal RNA in a suitable configuration for protein synthesis. By 1964 Waller (47) had fractionated proteins of 70 S ribosomes from several bacterial species into at least 24 distinct bands by electrophoresis on starch gels. However, the possibility remained that several of these bands were artifacts or aggregates formed during the purification process. Several

years later Traut *et al.* (46) isolated some of the proteins on carboxymethyl cellulose and obtained unique tryptic "fingerprints" and amino acid compositions for each protein tested. Kaltschmidt *et al.* (18) purified twenty 30 S proteins by means of preparative starch gel electrophoresis and showed that each protein had an individual tryptic "fingerprint" and amino acid analysis pattern.

Two problems have complicated the analysis of ribosomal proteins. The first has been the need to distinguish ribosomal proteins from contaminants. One way to determine this is to show the requirement of a certain protein for ribosomal function. Kurland *et al.* (22) prepared ribosomes free of supernatant proteins and other small contaminants by ammonium sulfate fractionation. Enzymes previously regarded as irreversibly bound to the ribosome were removed and yet ribosomes prepared by this technique were as active for *in vitro* protein synthesis as the crudest ribosomes available. The second problem was the need for rigorous evidence that each purified protein was a unique chemical species. Craven *et al.* (6) analyzed the amino acid composition of each of twenty one 30 S ribosomal proteins whose fractionation was described by Hardy *et al.* (14). They found eighteen unrelated proteins and two chemically similar proteins. Another approach was to show that each protein does not produce any immunological cross reaction with other proteins.

Stöffler and Wittmann (41) prepared antisera specific against each of the twenty one homogeneous ribosomal proteins from the 30 S subunits of *E. coli*, but were unable to detect any immunological cross reaction between any of the proteins. By these methods, the identities of forty nine *E. coli* ribosomal proteins, twenty from the 30 S subunit, have been demonstrated unambiguously (6, 9, 12, 14, 20, 29, 31, 33, 41, 46).

The first successful attempts to identify the function of individual ribosomal proteins were made by Traub *et al.* (44, 45). The 30 S subunits were dissociated by centrifugation in cesium chloride into "core" particles containing 16 proteins and 5 "split" proteins. The "split" proteins were then fractionated by phosphocellulose chromatography. 30 S subunits were then reconstituted from the purified or partially purified "split" proteins and "core" particles, but one "split" protein at a time was omitted from the reconstitution mixtures. Activity studies indicated that all five "split" proteins were necessary. Subsequently, Traub and Nomura (45) reconstituted ribosomes from (1) rRNA and (2) purified or partially purified protein components but again one protein at a time was omitted from the reconstitution mixtures. The assembled products were analyzed in two ways. First it was determined if a particle with a sedimentation coefficient similar to native 30 S particles was recovered.

When the particle was clearly disrupted by the omission of a protein, it was concluded that this protein was necessary for subunit assembly. Similarly, when the protein synthesizing functions of the reconstituted particle were deficient, it was concluded that the omitted protein was necessary for protein synthesis. These experiments yielded an assembly map of *E. coli* 30 S proteins (Fig. 1). The functions of the individual proteins have been reviewed most recently by Kurland (23). In order to simplify the literature concerning ribosomal proteins, several laboratories have agreed to use a single nomenclature based on the mobilities of the proteins during the electrophoretic fractionation by the two dimensional gel electrophoresis technique of Kaltschmidt and Wittmann (Fig. 2) (19, 52).

After it was determined that the *E. coli* ribosome was composed of an assortment of proteins, the 30 S proteins from different species were compared. Experiments by Huang and Sypherd (17) and Ozawa *et al.* (34) with ribosomal proteins from *E. coli* and several enteric bacteria showed that many of the 30 S proteins are indistinguishable by chromatographic and electrophoretic means. Tryptic fingerprints and amino acid analyses also showed no significant differences, or only minor differences, between paired proteins. Wittmann *et al.* (53) found that antisera prepared against 10 proteins from *E. coli* reacted to the same extent as with the homologous

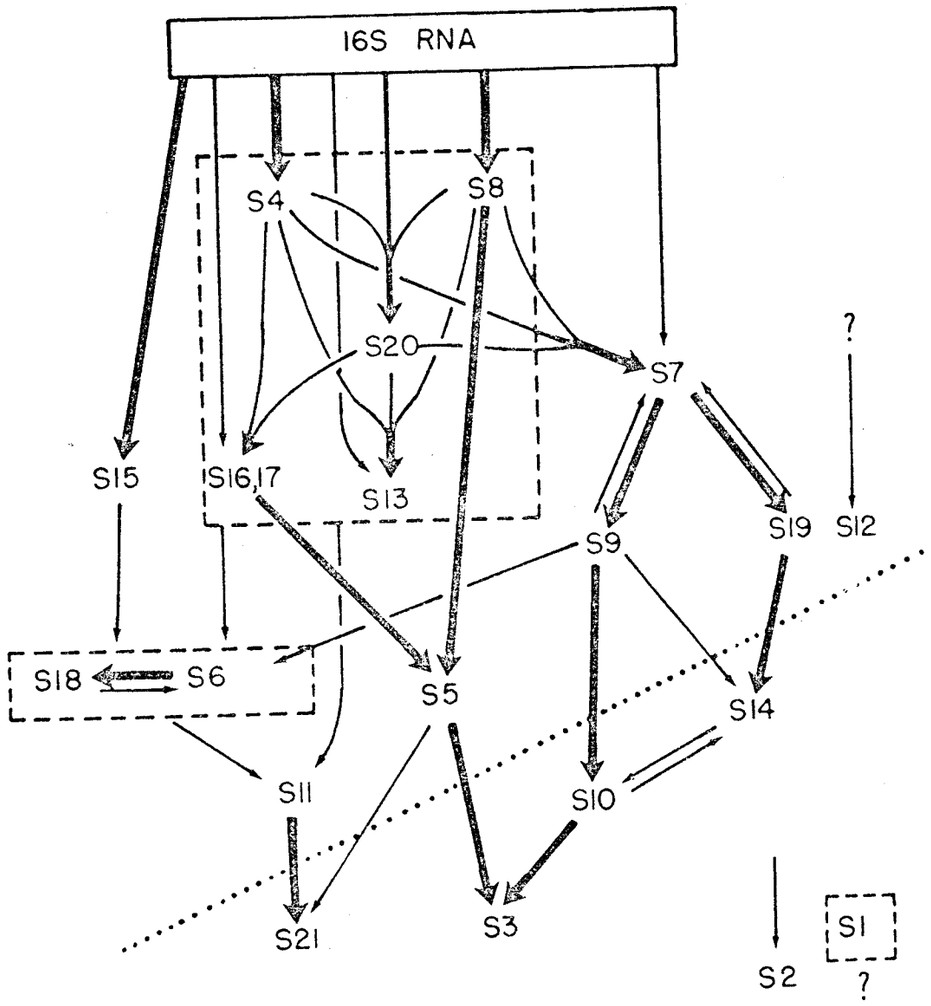

Fig. 1. Assembly map of 30 S ribosomal proteins. Arrows between proteins indicate the effect of a protein on another protein whose binding it helps. A thick arrow indicates a major contribution. The boxes (outlined in dashes) indicate the unit structural proteins. All proteins above the line of dots are present in the "core" particle. All below are split proteins. To avoid complication S 2 is not included and S 1 does not bind. This map is from the work of Mizushima and Nomura (28).

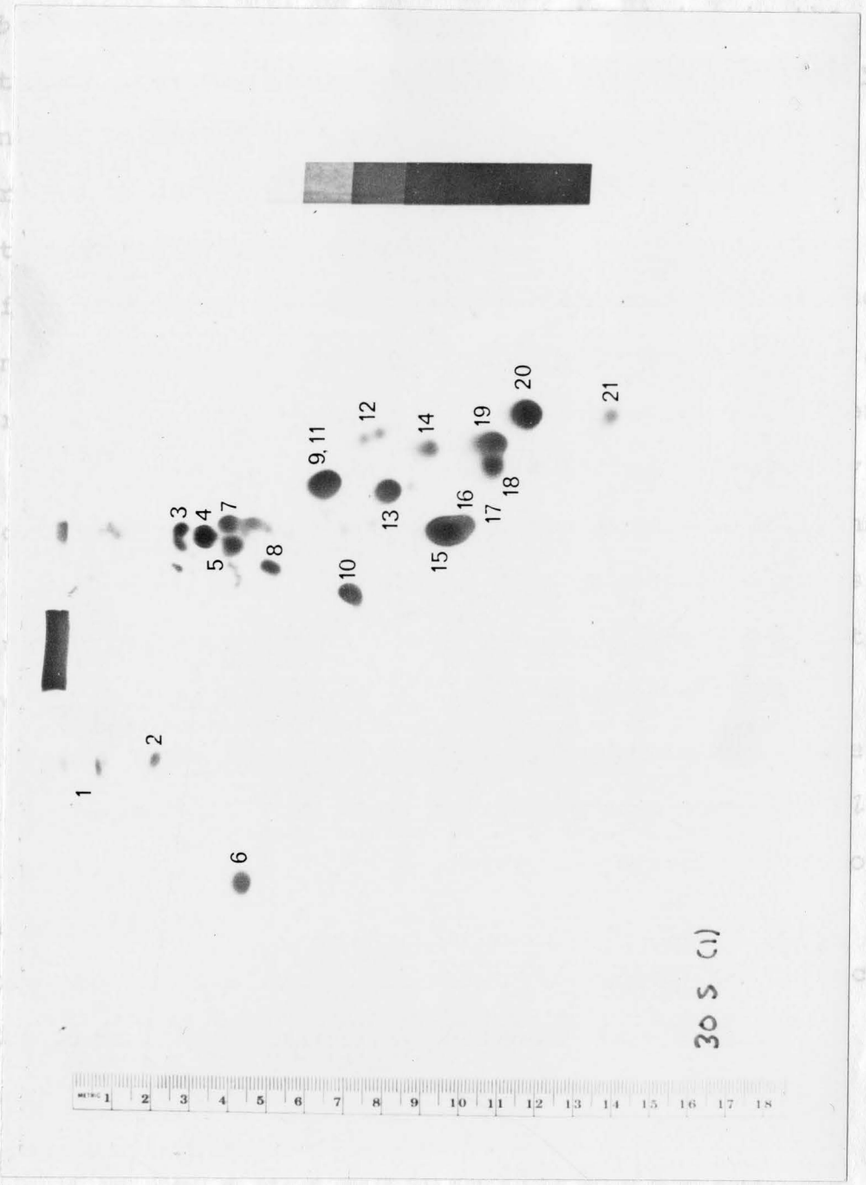

Fig. 2. A two dimensional separation of 30 S ribosomal proteins from *E. coli* in a polyacrylamide gel. The numbers correspond to the ribosomal protein nomenclature of Kaltschmidt and Wittmann (19, 51).

system, with 10 proteins from *S. typhimurium* and other *Enterobacteriaceae*. These extensive similarities in the chemistry of proteins suggested a great genetic similarity between the *Enterobacteriaceae* and a strong evolutionary pressure to conserve the basic pattern of RNA-protein or protein-protein interactions.

After Nomura *et al.* (32) had demonstrated that ribosomal proteins from genera as diverse as *Bacillus* and *Azotobacter* could replace *E. coli* proteins in reconstitution experiments, Ansley and Sypherd (1) fractionated *Bacillus* proteins by electrophoresis and compared their amino acid compositions with *E. coli* proteins. They found that *Bacillus* proteins were quite different from *E. coli* proteins, moreover, antisera prepared by Wittmann against *E. coli* proteins (53) did not show any cross reaction with *Bacillus* ribosomal proteins. However, Higo *et al.* (15) have recently shown that *Bacillus* proteins can replace *E. coli* proteins one-for-one in reconstitution experiments.

Our aim in this study has been to isolate and characterize *Bacillus* protein S 19 which cross reacts immunologically and functionally replaces *E. coli* protein S 19 (15) and to compare this protein with the *E. coli* S 19.

MATERIALS AND METHODS

Organism. A naturally occurring streptomycin resistant mutant of *Bacillus stearothermophilus* strain 799 obtained from Mr. John Dougherty, Institute for Enzyme Research, the University of Wisconsin was used in this study.

Conditions of culture. Bacteria were grown at 62 C in a modified L broth containing in g/l: 10.0, Tryptone (Difco); 5.0 Yeast Extract (Difco); 5.0, sodium chloride (NaCl); and 1.0 glucose. The pH was adjusted to 7.2 before autoclaving, and growth was monitored with a Klett-Summerson colorimeter fitted with blue filter #42.

One hundred fifty-liter cultures. *Bacillus* suspensions were inoculated into 10 100 ml Klett flasks containing 50 ml L broth, and incubated in a rotary water bath at 62 C until well into the logarithmic phase of growth (100 Klett Units). These cultures were then inoculated into 5 2 l Erlenmeyer flasks containing 1 l of L broth and incubated without auxiliary aeration in the rotary water bath at 62 C until mid log-phase.

At 75-100 Klett Units, this suspension was inoculated into a 50 gallon fermentor heated to 62.5 C containing 150 liters of L broth supplemented with an additional 0.5 g glucose/l. The fermentor was aerated with filtered compressed air at the rate of 1 ft^3/min with agitation at 75 rev/min.

Growth and pH were monitored by removing small aliquots aseptically at regular intervals. Oxygen consumption was monitored continuously with an oxygen probe. When log-phase growth had proceeded for a minimum of one generation time (about 20 min), aeration was increased to 2 ft^3/min and agitation to 100 rev/min. After the cells had grown to 150 Klett Units and the pH had dropped to 6.2, 300 ml of 1 N sodium hydroxide (NaOH) was added to neutralize the acid. At 250 Klett Units aeration was increased to 5 ft^3/min and agitation to 150 rev/min. Organisms were grown to a final turbidity of 300 Klett Units. 400 lb of crushed ice were poured into the fermentor to halt metabolism as quickly as possible. Within 10 min the temperature was lowered from 62 C to 7 C. The cells were harvested in a Sharples super centrifuge and frozen in 100 g packets at -20 C until use.

Preparation of ribosomes. All manipulations during ribosome extraction were performed at 0-4 C according to Tissieres *et al* (43). Cell-free extract was prepared by thawing 100 g cells and mixing with 200 g levigated alumina in a cold mortar. This mixture was hand ground for 4 to 5 min and extracted with 4 vol of TMA-I buffer containing: 10^{-2}M Tris (hydroxymethyl) aminomethane HCl (Tris), pH 7.8; 10^{-2}M magnesium chloride ($MgCl_2$); 3 x 10^{-2}M ammonium chloride (NH_4Cl) and 6 x 10^{-3}M 2-mercaptoethanol. One hundred µl

deoxyribonuclease (DNase) (0.25 mg/ml, Sigma) was added and the mixture stirred for 10 min. This mixture was centrifuged at 10,000 x g for 30 min; the supernatant decanted and saved. The pellet containing alumina, broken cells, and trapped ribosomes was resuspended in 2 vol TMA-I and then recentrifuged. The two resulting supernatants were pooled and centrifuged at 30,000 x g for 30 min in order to sediment cell membrane fragments. The top 4/5 of the resulting supernatant was decanted. This supernatant termed "crude extract" was then centrifuged at 30,000 x g for 8 hr in a Spinco 30 rotor, to sediment the ribosomes.

The resulting crude brown-colored ribosomal pellet was resuspended in TMA-I buffer and layered upon a sucrose-salt cushion containing, according to Staehelin and Maglott (40): 1.1 M Sucrose; 0.5 M NH_4Cl; 10^{-2}M magnesium acetate (CH_3COOMg); 5×10^{-4}M ethylenediamine trichloroacetic acid (EDTA); and 2×10^{-2} M Tris-HCl, pH 7.5. This was centrifuged in a Spinco 42 rotor at 50,000 x g for 26 hr, in order to strip the ribosomes of any extraneous material. The resultant clear, colorless ribosomal pellet was then resuspended in TMA-I and frozen at -80 C until use.

Estimation of ribosome concentration. Ribosome concentration estimates were based on a specific extinction coefficient at 260 nm: $E^{1\%}_{1cm} = 160$ for *E. coli* ribosomes, calculated by Tissieres *et al* (43). RNA and protein estimations indicate that 42% of the ribosome is protein and 58% is RNA (43).

Preparation of 30 S subunits. Frozen ribosomes were thawed and dialyzed for up to 8 hr against TMA-II (same as TMA-I except that the $MgCl_2$ concentration is 3×10^{-4}M), in order to dissociate ribosomes into their component 30 S and 50 S subunits. An exponential sucrose density gradient in TMA-II was formed with a Beckman Model 141 Gradient pump in a Spinco Ti-15 zonal rotor. The sucrose gradient which is described in detail by Eikenberry *et al* (11), extends from 7.4% (w/w) (1.029 g/cm^3) to 38% (w/w) (1.171 g/cm^3) in a volume of 900 ml. The dialyzed sample containing typically 20-25,000 A_{260} dissociated ribosomes in a sucrose gradient (7.4% to 0%), was introduced onto the separating gradient and followed by an overlay of 700 ml TMA-II.

The rotor was operated at 31,000 rev/min for 10 hr at 4 C. Unloading was accomplished by displacement of the gradient with 60% sucrose. After discarding the first 1000 ml, fractions of 15 ml were collected. The absorbance at 260 nm was determined and peak fractions were pooled.

Mg concentration was adjusted to 10^{-2}M and subunits were recovered by precipitation with 0.7 vol ethanol (EtOH) overnight. The precipitated subunits were collected by centrifugation at 6,000 x g for 1 hr and dialyzed exhaustively against TMA-I to remove sucrose and EtOH. Subunits were then frozen at -80 C until use.

30 S purity assay. To examine the 30 S subunit fraction for contamination with the larger subunit, analytical density

gradients were performed in a Spinco Ti-65 swinging bucket rotor using 5% to 20% sucrose density gradients in TMA-II. From each, 30 S pool, 4 A_{260} were layered onto the analytical gradients. Selected gradients received radioactive 50 S and 30 S subunits prepared from *E. coli* grown in $^{35}SO_4$. Centrifugation was performed at 50,000 rev/min for 90 min. Three drop/tube fractions were collected, diluted to 1 ml with H_2O, and the A_{260} of each fraction determined. To each radioactive fraction, 1 ml of albumin solution (1 mg/ml) was added to facilitate precipitation. One ml 10% trichloroacetic acid (TCA) was then added and this suspension was poured rapidly onto Millipore filter pads (0.45 μm porosity). The tube was then rinsed twice with 10 ml of 5% TCA and this solution was poured onto the pad.

The pads were then dried under an infrared lamp and counted for 5 min in a low background scintillation counter (Nuclear Chicago). Plots of A_{260} and specific activity versus fraction number were made to determine purity.

Extraction of total 30 S protein by the lithium chloride-urea method. After purity had been ascertained, subunits were mixed with an equal volume of 8 M urea-6 M LiCl (25) and allowed to stand on ice for about 48 hr. Since it was necessary for the ribosomal components to be in contact with the urea and lithium chloride for an extended period of time, the urea and lithium chloride were prepared with

great care. A saturated solution of urea was stirred with Amberlite (MB-1) for 1 hr at room temperature. The Amberlite was removed by filtration through a frittered glass filter. Activated charcoal (Norit A) was then stirred into the urea for at least 1 hr. The charcoal was then removed by filtration through Whatman No. 2 paper. This purified urea solution was then allowed to crystallize overnight in the cold room. Urea crystals were removed, washed with chloroform and dried *in vacuo*. A 10 M solution of lithium chloride was purified by adding 4 g/l Norit A and stirring for 1 hr. The charcoal was removed on Whatman No. 2 paper. A solution of 8 M urea-6 M LiCl was then prepared.

After standing for 68 hr the 30 S-LiCl-Urea mixture had settled into 2 phases, (1) a white precipitated lower zone of RNA and (2) a clear supernatant above it containing the 30 S ribosomal proteins. These zones were centrifuged separately at 8,000 x g for 20 min to pellet the RNA. The protein solution was decanted and A_{260}:A_{280} was determined to assay for RNA contamination.

The protein solution was then dialyzed against 20 vol 6 M urea-methylamine-phosphate buffer (UMP) (preparation described below) to lower the salt concentration from 3 M to that of the chromotography starting buffer (0.15 M). After 8 hr dialysis, the protein solution was dialyzed against 0.15 M LiCl-UMP pH 8.00, until loading on the phosphocellulose column.

Phosphocellulose chromatography of ribosomal proteins at pH 8.00. Buffer preparation. Chromatography was performed in phosphate buffered 6 M urea. Reagent grade urea (Mallinckrodt) has a high A_{230} relative to water which reduced to a low level by decolorizing with activated charcoal (Norit A). To a solution of 6 M urea, 50 g/l Amberlite (MB-1, Mallinckrodt), was added and the solution stirred for 1 hr in the cold. The Amberlite was then removed by filtration. Activated charcoal was then added to the solution and stirred for at least 1 hr in the cold. The charcoal was then carefully removed by filtration on Whatman No. 2 paper. The decolorized urea was then used to prepare the standard urea-methylamine-phosphate (UMP) buffer containing in 6 M urea: 0.05 M phosphoric acid; 0.012 M methylamine and 50 µg/l 2-mercaptoethanol. Mercaptoethanol prevents disulfide bond formation; the methylamine is present as a scavanger for cyanate (13). The pH was adjusted to exactly 8.00 with phosphoric acid (85%, Eastman) and methylamine (40% in water). LiCl-UMP buffers, 0.20 M and 0.6 M, were made by adding the appropriate volume of 10 M LiCl to the standard UMP buffer and adjusting to pH 8.00.

Phosphocellulose preparation. Two hundred g standard capacity Mannex-P phosphocellulose (0.9 meq/g) were suspended in distilled water, stirred briefly, allowed to settle for 15 min and then decanted to remove fines. This process

was repeated several times until about 50% of the material had been discarded as fines. One l of 0.1 N NaOH was added and stirred for 10 min. This mixture was then rapidly filtered through a Whatman No. 2 paper fitted-Buchner funnel under vacuum. About 9 l water were then added to wash away traces of NaOH. After suspending in 1 l of H_2O the pH was adjusted to 8.00 with methylamine and phosphoric acid. After 30 min the water was filtered off and the phosphocellulose was placed in 0.15 M LiCl-UMP equilibration buffer and the pH adjusted to 8.00. This solution was allowed to stand overnight to equilibrate the phosphocellulose to pH 8.00. Just prior to column packing, the phosphocellulose was degassed for 5 min.

Column packing. A 26 x 150 cm column was packed with phosphocellulose under gravity at a flow rate approximately 2 times that required for development of the chromatogram. After packing, the column was flushed overnight at 4 C with 0.15 M LiCl-UMP (pH 8.00) or until the pH of the effluent was 8.00.

Column loading and gradient elution. All operations were performed at 4 C. The protein sample was allowed to run onto the column and then followed by a void volume of 0.15 M LiCl-UMP, pH 8.00 buffer. When this had run onto the column, 6 l of a 0.2 M to 0.6 M LiCl-UMP pH 8.00 linear gradient was started in order to elute the proteins. After

the gradient had run onto the column it was followed by one void volume of 1 M LiCl-UMP (pH 8.00) in order to elute any proteins held on the column. A constant flow rate of 40 ml/hr was maintained throughout these operations and fractions of 200 drops per tube were collected in an LKB fraction collector.

Phosphocellulose chromatography at pH 6.5. Chromatography was performed in 6 M UMP buffer as described above except at pH 6.5. Sample and phosphocellulose were equilibrated (separately) to 0.15 M LiCl in UMP pH 6.5.

A 20 x 60 cm column was packed as above and after equilibration to pH 6.5, the sample was loaded onto the column. Four l of a 0.25 M to 0.45 M LiCl-UMP (pH 6.5) gradient were then run onto the column to elute the proteins.

One M LiCl-UMP (pH 6.5) buffer was then run onto the column to elute any remaining protein. As above, 200 drop/tube fractions were collected at a flow rate of 40 ml/hr.

Protein Determination. Protein (tyrosine) was estimated by a modification of the Lowry method (26). Solution A containing 1 ml 2% copper sulfate ($CuSO_4 \cdot 5H_2O$) 1 ml 4% sodium potassium tartarate and 48 ml of 3% sodium carbonate (Na_2CO_3) in 0.1 N NaOH was made. Folin-Ciocalteu reagent was diluted 1:3 with water just before use.

To a 0.5 ml protein sample, 5 ml of solution A was added, mixed, and allowed to stand at room temperature for 10 min after which 0.5 ml of diluted Folin reagent was very rapidly added and mixed well. After 30 min A_{750} was read.

This procedure was used to determine lysozyme, bovine serum albumin and total ribosomal protein standards. During large scale preparations A_{230} was used to estimate protein.

Protein concentration. After phosphocellulose chromatography, fractions were fairly dilute, therefore it was necessary to concentrate them. This was done either by ultrafiltration or on small phosphocellulose columns.

Concentration by ultrafiltration was done with the Amicon #52 unit. UM-2 membranes, having a pore size of 10 Å and capable of excluding substances M.W. 1000 or greater, were used.

Concentration by phosphocellulose column was done in the following manner. Small columns (2 x 15 cm) were packed with phosphocellulose at the appropriate pH. The protein fraction was diluted 3 or 4 fold to lower the salt concentration, and loaded onto the small column. Two to three l of dilute sample (100 mg protein) were applied to the column and eluted in about 10 to 15 ml with 2 M LiCl in urea buffer.

Sephadex gel filtration. Concentrated fractions were separated by size on a Sephadex G-100 column in 0.15 M LiCl-UMP pH 8.00. 10 ml fractions were collected and A_{230} was determined.

Protein desalting. After Sephadex fractionation and phosphocellulose concentration, protein was desalted by

layering onto a column of Bio-Gel P-2 in 50% acetic acid. Fractions of 200 drops/tube were collected and A_{280} determined. Peak fractions were pooled, frozen and lyophilized. After the first lyophilization the protein was resuspended in 20 ml distilled water, shell frozen, re-lyophilized, and stored dessicated at room temperature until use.

Acrylamide gel disc-electrophoresis. One dimensional system for analysis of ribosomal proteins. Disc-electrophoresis of ribosomal proteins at pH 4.5 in urea was carried out according to Leboy (25). The solutions contained: Solution A: 12 ml 2 N potassium hydroxide (KOH), 8.6 ml glacial acetic acid, 2.0 ml N, N,N',N'-tetramethylethylenediamine (TEMED), 24 g urea and distilled water to make 50 ml. Solution B: 24 ml 1 N KOH, 1.44 ml glacial acetic acid, 0.23 ml TEMED, 24 g urea, and distilled water to make 50 ml. Solution C: 6.65 g acrylamide (Eastman), 0.1 g N,N-methylene-bisacrylamide (MBA, Eastman), 24 g urea, and distilled water to make 50 ml. Solution D: 2.5 g acrylamide, 0.625 g MBA, 24 g urea, and distilled water to made 50 ml. Solution E: 1.0 mg riboflavin in 8 M urea, prepared fresh daily. Stock buffer for electrophoresis: 31.3 g β-alanine, 8.0 ml glacial acetic acid and distilled water to 1 l. The pH of the buffer was adjusted to 4.5 and the solution was stored at 4 C. Prior to use the stock solution was diluted five-fold with distilled water.

The running gels were prepared by mixing the stock solutions in the following proportion: 1 part A, 6 parts C, 1 part F, and 0.5 mg potassium ferricyanide [$K_3Fe(CN)_6$]/8 ml of mixture. For the spacer gels, the mixture consisted of 1 part B, 4 parts D, 1 part E, and 2 parts 8 M urea.

Gels were polymerized in glass tubes, 100 mm long with an inner diameter of 6 mm. The glass tubes were filled with 1.5 ml running gel, overlayered with water, and allowed to polymerize about 10 cm from a fluorescent light source for 30 min. Following polymerization of the running gel the water overlay was removed and the top of the running gel was rinsed with spacer gel. The spacer gel (0.2 ml) was polymerized using the above procedure. After polymerization of the spacer gel, a 100 µl sample (containing 200-500 µg protein) was mixed with 100 µl of spacer gel solution, 1 µl of 0.1% pyronine red to serve as the tracking dye, and a drop of 14.3 M mercaptoethanol added.

A constant current of 2.5 mA per tube was applied. The anode was placed in the upper chamber and electrophoresis performed for approximately 3 hr at 4 C, until the tracking dye reached the bottom of the tube. The run was then terminated. The gels were removed from their respective glass tubes and stained in amido black (1% w/v in 7.5% acetic acid) for 1 hr. Gels were then rinsed of excess dye with water and electrophoretically destained.

Procedure for 2-D acrylamide gel electrophoresis of ribosomal proteins. 1-D gel, pH 8.6. The apparatus and procedure of Kaltschmidt and Wittmann (19) was used for the 2-D acrylamide gel electrophoretic analysis of ribosomal proteins. The 1-D 8% separation gel consisted of the following components in g: 8.0 acrylamide, 0.3 MBA, 3.2 boric acid, 0.8 Na_2 EDTA, 4.85 Tris, 0.3 ml TEMED, 36 urea, and distilled water to 99.0 ml. To catalyze polymerization 1.0 ml of 3% ammonium persulfate was added. The 4% spacer gel contained in g: 40.0 acrylamide, 2.0 MBA, 3.2 boric acid, 0.85 Na_2 EDTA, 0.6 ml TEMED, 480 urea, and distilled water added to make 1 l. This solution was then polymerized by the addition of 0.5 mg riboflavin and 3 mg ammonium persulfate dissolved in 1 ml distilled water. The overlay buffer consisted of the following in g: 0.32 boric acid, 0.085 Na_2 EDTA, 16 urea, and distilled water added to make 100 ml. The electrode buffer consisted of the following in g: 7.2 Na_2 EDTA, 28.8 boric acid, 43.65 Tris base, 1,080 urea, and distilled water added to make 3 l.

When the acetic acid extraction procedure for isolation of ribosomal proteins was used, the protein samples were dialyzed against 20% acetic acid for 2 hr, and then dialyzed for 2 hr against 500 ml of unpolymerized spacer gel solution which contained all reagents listed above except TEMED, riboflavin, and ammonium persulfate. The samples were then

above. The tubes were placed in the electrophoresis apparatus with the anode in the upper chamber. Ninety volts was applied or about 2.5 mA tube (10 tubes) and electrophoresis was performed for 37 hr at room temperature. After electrophoresis was complete, the gels were removed from the tubes.

2-D gel, pH 4.6. The 2-D separation gel consisted of the following components in g: 306 acrylamide, 8.5 MBA, 88.9 ml glacial acetic acid, 16.3 ml 5 N KOH, 9.9 TEMED, 612 urea, and distilled water to make 1635 ml (for 5 gels). The dialysis buffer consisted of the following components in g: 960 urea, 1.48 ml glacial acetic acid, 4.8 ml 5 N KOH, and distilled water to make 2 l. The electrode buffer (for 5 samples) consisted of the following components in g: 168 glycine, 18 ml glacial acetic acid, and water to 12 l.

The 1-D gels were dialyzed against the dialysis buffer (750 ml three times with a change of buffer each hr). The 2-D gel was degassed by bubbling N_2 through it for 5 min followed by suction for twenty min with continuous stirring in an ice bath. The gel solution and 2-D apparatus was then put in to the cold room and permitted to equilibrate at 4 C. When both the gel solution and 2-D apparatus had equilibrated, the plastic base was filled with the plug gel. To polymerize the plug, 19 ml of 8% ammonium persulfate solution was mixed with 550 ml of gel solution and poured into the plastic base. The gel was then overlayered with 30-40 ml water. The

2-D gel apparatus was then seated into place. After the plug gel polymerized, the water was removed from the 2-D gel by inserting rolled paper strips into the gel chambers. The 1-D gels were then positioned into the V-shaped crevice in the horizontal position. To polymerize five 2-D gels, 1,110 ml of separation gel were mixed with 38 ml of 8% ammonium persulfate and poured into the vertical 2-D chambers, high enough to surround the 1-D gel but not to cover it. Air bubbles were then removed from under the 1-D gel by gently lifting the gel at either end. As the gel was polymerizing, additional amounts of gel were added so that the level of gel solution never fell below that of the 1-D gel. After the 2-D gel had polymerized, the gel plug in the plastic base was removed with the aid of a spatula and the 2-D apparatus was lifted out of the base. The excess gel block formed at the bottom of the apparatus and gel that had been spilled on the bottom of the upper buffer chamber were removed. The gel box was then rinsed in distilled water and then put in place in the lower buffer chamber. Samples were electrophoresed at 105 volts and 480 mA for 26 hr with the cathode in the upper chamber. Following electrophoresis, the 2-D gel slabs were removed, placed onto racks, and stained in 12 l of amido black (0.55% w/v in 7.5% acetic acid) for 5 min. The slabs were washed for 24 hr in continuously flowing tap-water and then destained in 1-1.5% acetic acid until the gels cleared.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis for molecular weight determination. SDS-gel electrophoresis was carried out according to Weber and Osborn (51). The solutions contained: Solution A: 2 ml 1% SDS, 0.2 ml 2-mercaptoethanol, 0.2 ml 1 M phosphate buffer pH 7.1, 2 ml glycerol and water to 10 ml. Solution B: 80 ml 1 M phosphate buffer pH 7.1, 0.4 ml TEMED, 10 ml 1% SDS and water to 100 ml. Solution C: 30 g acrylamide, 1.5 g MBA, 10 ml 1% SDS and water to 100 ml. Solution D: 60 mg ammonium persulfate and 1% SDS to 10 ml. Solution E: 1% SDS solution. Electrophoresis buffer containing 0.1 M phosphate pH 7.1 and 1% SDS was freshly prepared for each run.

Gels were prepared by mixing 1 part B, 4 parts C and 2 parts E in a small aspirator bottle, and degassing for 5 min. After chilling, 1 part D was added, and the solution was poured into glass tubes (6 x 73 mm) and overlayered with water. Gels were polymerized for 30 min or until slightly opaque. Protein sample (30 µg in 20 µl) was mixed with 30 µl A and heated at 65 C for 10 min in a stoppered tube. 5 µl 0.2% Bromophenol blue was added as a tracking dye. Tubes were filled with electrophoresis buffer and underlayered with sample. The two compartments of the apparatus were filled with electrophoresis buffer and electrophoresis was performed at a constant current of 15 mA/gel, with the anode

in the lower chamber, for about 4 hrs. After electrophoresis, the gels were removed from the tubes by air pressure with a syringe. The gel length, and tracking dye mobility were measured and a fine gauge piece of wire was inserted through the tracking dye band. Gels were stained for 2 hrs in a solution containing 1.25 g Coomassie brilliant blue, 454 ml 50% methanol, and 46 ml glacial acetic acid. Gels were destained by diffusion in a solution containing: 75 ml acetic acid, 250 ml methanol and 675 ml water. Mobility was calculated as: mobility = distance of protein migration/ length of gel after destaining X length before staining/ distance of tracking dye migration. Plots of logarithm of molecular weight versus mobility, were made with several proteins of known molecular weight which had been run not only in the same gel with the sample but also separately. In this way the molecular weight of the protein sample could be estimated.

N-terminal amino acid sequence determination by automated Edman procedure. Sequencing was performed with a Beckman 890 B sequenator adapting the principles of Edman and Begg (10). The following reagents and solvents were used in the operation of the sequenator: Reagent 1: 5% (v/v) phenylisothiocyanate (PITC) in heptane, Reagent 2: Quadrol (N,N,N',N'-tetrakis [2-hydroxypropyl] ethylenediamine), Reagent 3: heptafluorobutyric acid, Solvent 1: benzene,

Solvent 2: ethyl acetate, Solvent 3: chlorobutane.

150-250 nM of protein was dissolved in formic acid and placed in the rotating sequenator reaction cup. 10 µl butanethiol was added. The formic acid was evaporated under vacuum. The resulting film of protein was coupled in a nitrogen atmosphere with phenylisothiocyanate in Quadrol buffer at 55 C. Non-protein components were removed by a "rough" vacuum followed by a "fine" vacuum and finally by extraction with organic solvents. The dried protein film was then exposed twice to anhydrous heptafluorobutyric acid and the amino-terminal residue extracted twice in chlorobutane as a phenylthiazolinone derivative. At this point one sequenator cycle was complete and a new double cleavage cycle began as detailed in Table 1.

Analysis of sequenator products. Fractions recovered from the sequenator were delivered to the fraction collector in the form of the phenylthiazolinone. Before analysis in the gas chromatograph, the amino acid had to be converted to the corresponding phenylthiohydantoin (PTH) amino acid.

Fractions were converted by adding 0.2 ml of 1.0 N HCl to each dried tube, in an 80 C bath for 10 min under a nitrogen atmosphere. Ethyl acetate (0.7 ml) was then added, mixed and centrifuged to separate the liquid components. The top layer containing all PTH amino acids (except arginine, histidine and cysteic acid) was removed and the ethyl acetate extract was reduced to dryness at room temperature with nitrogen gas.

TABLE 1: SEQUENCER PROGRAM STATEMENTS

Program Step		Step Time (sec)
1	Stop slew	2
2	Cell pressurize	6
3	Delay	2
4	R_1 + R_2 vent	10
5	R_1 + R_2 pressurize	30
6	Delay	2
7	R_1 deliver (effluent to waste open)	4
8	Restricted vacuum	40
9	Cell pressurize	6
10	N_2 dry	20
11	Delay	2
12	R_2 pressurize	4
13	R_2 deliver (effluent to waste open)	16
14	Coupling reaction	120
15	Coupling reaction	840
16	Coupling reaction	840
17	S_2 vent	30
18	S_2 pressurize	30
19	S_2 deliver (effluent to waste open)	4
20	Restricted vacuum	300
21	Rough vacuum	300
22	Fine vacuum	300
23	Delay	2
24	Cell pressurize	6
25	S_1 vent	30
26	S_1 pressurize	30
27	S_1 deliver (effluent to waste open)	300
28	N_2 dry	200
29	S_2 vent + restricted vacuum	30
30	S_2 pressurize + restricted vacuum	180

SEQUENCER PROGRAM STATEMENTS (cont.)

Program Step		Step Time (sec)
31	Rough vacuum	30
32	Delay	2
33	Cell pressurize	6
34	S_2 deliver (effluent to waste open)	800
35	Delay (effluent to waste open)	60
36	Restricted vacuum	60
37	Rough vacuum	40
38	Fine vacuum	360
39	Delay	2
40	Cell pressurize	6
41	R_3 vent	10
42	R_3 pressurize	10
43	R_3 deliver (effluent to waste open)	7
44	First cleavage reaction	180
45	Restricted vacuum	60
46	Rough vacuum	40
47	Fine vacuum	140
48	Delay	2
49	Cell pressurize	6
50	S_3 vent + FC step + FC vent	30
51	S_3 pressurize	30
52	S_3 deliver--collect (effluent to FC open)	150
53	Delay (effluent to FC open)	40
54	Restricted vacuum	60
55	Rough vacuum	20
56	Fine vacuum	60
57	Delay	2
58	Cell pressurize	6
59	R_3 vent	10
60	R_3 pressurize	10

SEQUENCER PROGRAM STATEMENTS (cont.)

Program Step		Step Time (sec)
61	R_3 deliver (effluent to waste open)	7
62	Second cleavage reaction	120
63	Restricted vacuum	60
64	Rough vacuum	40
65	Fine vacuum	140
66	Delay	2
67	Cell pressurize	6
68	S_3 vent	30
69	S_3 pressurize	30
70	S_3 deliver (effluent to waste open)	150
71	Delay (effluent to waste open)	40
72	Restricted vacuum	60
73	Rough vacuum	20
74	Fine vacuum	800
75	Start slew (initiate new cycle)	
76	Fraction dry, FC vacuum + Fine vacuum	980
77	Fraction dry, FC vacuum + Fine vacuum	980
78	Fraction dry, FC vacuum + Fine vacuum	980
79	Conditional stop, Fine vacuum	980

This residue was then dissolved in 20 µl ethyl acetate, mixed thoroughly and used as the sample in gas chromatography.

If the residue in question was thought to be histidine, arginine or cysteic acid, then the bottom aqueous layer was used. The pH was raised by adding 0.2 ml of 1 M dibasic sodium phosphate (Na_2HPO_4). Ethyl acetate (0.7 ml) was added, mixed and centrifuged to separate the liquid components. The top layer was removed and the ethyl acetate extract reduced to dryness under nitrogen. The residue was then dissolved in 20 µl methanol and used for gas chromatography.

If the residue was considered to be arginine gas chromatography was unsatisfactory for detection and some other method used.

If the residue in question was considered to be aspartic or glutamic acid, silylation was necessary before the sample could be injected into the gas chromatograph (G.C.). In this case 25 µl sample was mixed with 25 µl of BSA (N, O-bis-trimethylsilylacetamide). This was reacted at 80 C for 1 min and the resultant silylated derivative (TMS) was then used as G.C. sample.

Samples (1-5 µl) containing 5-40 nM of the PTH-amino acid were injected into a Beckman G.C. 45 using a silylated column (2 mm x 1.2 m) containing SP-400 resin. The gas flow was 140 cm^3/min of helium and the instrument was programmed for a linear temperature rise from 190 to 290 C.

Amino acid analysis. Aminoethylation. Since cysteine is labile to acid hydrolysis, the protein was reacted with ethylenimine to convert cysteine residues into S-(2-aminoethyl) cysteine, a more stable derivative.

Four nM of protein were weighed and dissolved in 1 ml of UMP. 300 µl of 3 M Tris pH 8.6, and 50 µl of 2-mercapto-ethanol were added and vortexed. Protein was reduced under nitrogen for 5 hr.

After 5 hr, 50 µl ethylenimine was added and the reaction allowed to proceed for 10 min. Another 50 µl ethylenimine was added and after another 10 min a final 50 µl aliquot of ethylenimine was added.

After dialysis against 1200 vol of 0.6% acetic acid overnight at 4 C the sample was ready for hydrolysis.

Hydrolysis. After dialysis the samples were placed in scrupulously clean borosilicate (8 x 150 mm) hydrolysis tubes. These were covered with glassene paper (Lilly, Indianapolis), frozen, and lyophilized overnight. 60 ml of doubly distilled 6 N HCl (BP 107-108) and 2 ml thioglycolic acid were placed in a 1000 ml beaker. 2 ml of 6 N HCl was added to each hydrolysis sample tube and these were placed in the 1000 ml beaker and covered with a crystallization dish. This assembly was then placed in a vacuum dessicator and placed under vacuum for 15 min. It was then flushed with nitrogen several times and placed under vacuum again for 15 min.

The samples were then hydrolyzed for 20 hr at 110 C in an autoclave. After hydrolysis, the HCl was removed by rotary evaporation and each sample suspended in 2.2 ml of amino acid dilution buffer (Pierce, Rockford).

Amino Acid Analysis. Analyses were performed on a JEOL-AH6 amino acid analyzer utilizing a 2 column system (39). Chromatograms were hand or machine integrated and concentrations were determined by the use of standards.

RESULTS

1. Culturing of *Bacillus stearothermophilus*.

Bacillus was streaked onto plates containing L broth and 1.5 g/l agar, supplemented with 1 mg/l streptomycin (L-Sm). Organisms were picked from an individual colony restreaked onto L-Sm plates and incubated at 65 C.

Gram stain showed the presence of gram positive rods; phase contrast microscopy indicated the presence of motile, spore forming rods. This organism was thus characterized as a gram positive, motile, spore forming rod which grows at 65 C -- *Bacillus stearothermophilus*.

Individual colonies were inoculated into flasks containing 50 ml L-broth. They were placed in a 62 C rotary water bath and growth was followed. When cultures had reached late log-phase (100 Klett Units) they were inoculated into 2 l flasks containing 1 l L-broth. These were incubated at 62 C until mid log-phase. These flasks were then used to inoculate the 50 gallon fermentor containing 150 l of L-broth at 62.5 C. Since it was vital that growth be halted in mid log-phase, in one initial fermentation cells were allowed to grow into stationary phase. The growth curve for *Bacillus stearothermophilus* in the 50 gallon fermentor is shown in Fig. 3. From this growth curve we were able to estimate mid log-phase and in all succeeding fermentations growth

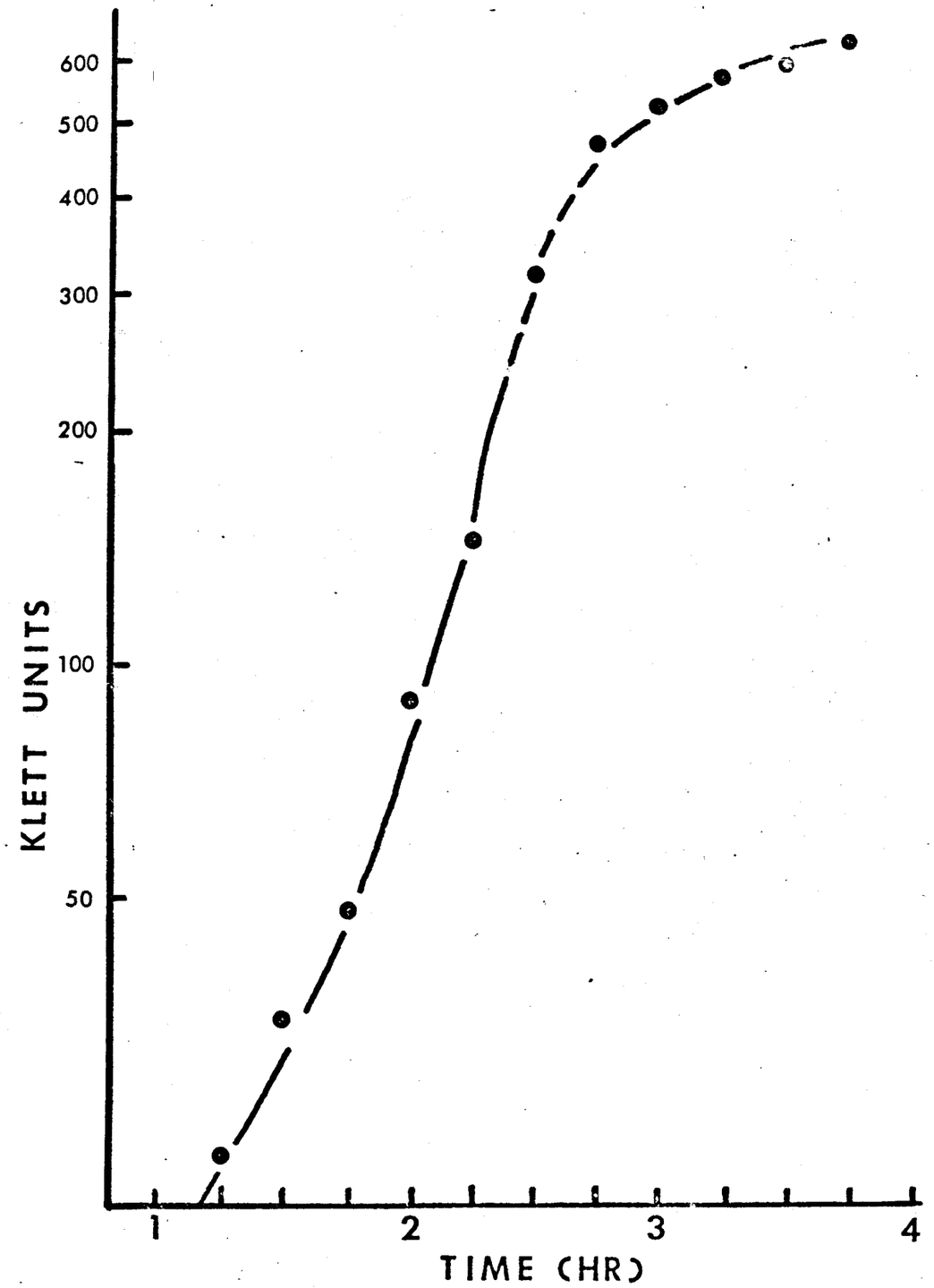

Fig. 3. 150 liter Fermentor growth curve of *Bacillus stearothermophilus*. Growth conditions are described in the text. Generation time of the organism is about 20 min. Growth was halted when turbidity reached the end of the solid line.

was allowed to proceed only until mid log-phase as is represented by the solid line in Fig. 3. Cells from six fermentor batches were accumulated to obtain 2.4 kg (wet weight) of cells.

2. Zonal centrifugation. Salt-washed ribosomes prepared as described above, were dialyzed against TMA-II (3×10^{-4} M Mg^{++}) in order to dissociate the ribosomes into the 50 S and 30 S subunits.

Varying amounts of ribosomes (ranging from 18,000 to 40,000 A_{260}) were layered onto zonal gradients. It was found that up to 20,000 A_{260} of ribosomes could be processed with no cross contamination between 30 S and 50 S peaks as shown in Fig. 4. When increasing quantities of ribosome were used, the valley between the two peaks became higher and fractions had to be cut very judiciously to avoid cross contamination. A total of 26 zonal runs were performed, yielding 101,000 A_{260} of 30 S from 430,000 A_{260} ribosomes.

Since the volume of the pooled 30 S zonal fractions was about 250 ml, subunits were concentrated by ethanol precipitation. More than 98% of the A_{260} were recovered by this method.

3. 30 S purity assay. Analytical sucrose density gradients were employed as described above. Plots were made of specific activity and A_{260} versus fraction number. A plot of specific activity (counts/min) gives 3 major peaks

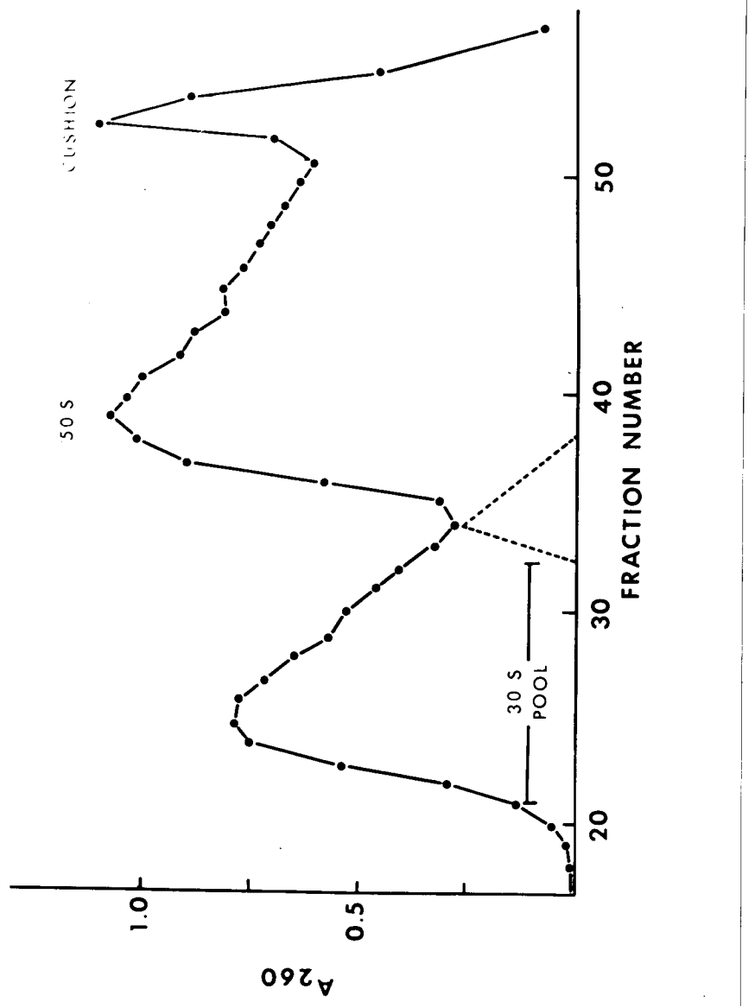

Fig. 4. Separation of 50 S and 30 S subunits by zonal centrifugation. Zonal preparation contains 18,000 A_{260} ribosomes. 5200 A_{260} of 30 S subunits are in the 30 S pool.

as shown in Fig. 5. These correspond to 50 S and 30 S subunits and 5 S RNA. The plot of A_{260} shows one major peak corresponding to the 30 S activity peak and a slight shoulder in the 43 to 50 S region. Successful chromatographic fractionation requires a contamination of less than 5% with the 43 or 50 S subunit. Hand integration of the A_{260} plots gave an estimation of 3% contamination.

4. Extraction of total 30 S protein by the Lithium Chloride-Urea method. Three hundred and twenty-two ml of 30 S in TMA-I, at a concentration of 314 A_{260}/ml were extracted with an equal volume of 8 M urea-6 M LiCl as described above. The protein was extracted for 68 hrs and centrifuged to pellet the precipitated RNA. Fifty µl of the resulting protein supernatant was diluted to 1 ml with UMP and absorbance at 260 nm and 280 nm was determined. The ratio of A_{260} to A_{280} was 1.11. Since the extinction at 260 and 280 is known for both RNA and protein a nomograph has been constructed. A ratio of 1.1 indicates an RNA contamination of 6%, a value slightly higher than the 5% which is usually acceptable for chromatography.

5. Phosphocellulose chromatography at pH 8.00. After dialysis, as described above, the 865 ml of total 30 S protein sample was separated into 2 equal aliquots. These were loaded onto two phosphocellulose columns at pH 8.00, and chromatography proceeded as described above. Seven

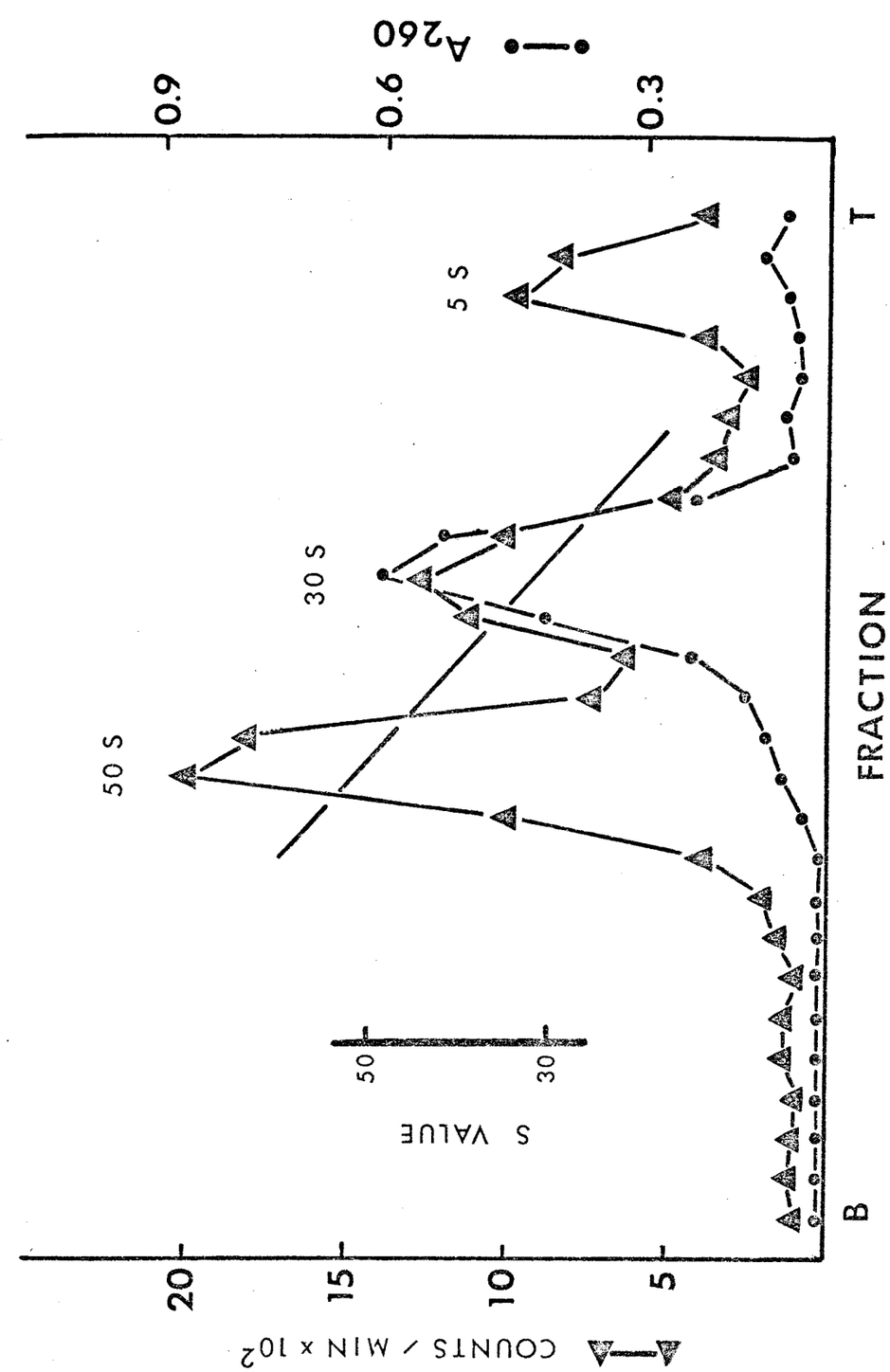

Fig. 5. Analytical sucrose density gradient of 30 S zonal pools. 4 A_{260} of *Bacillus* 30 S from a zonal preparation (O---O) and 35 S labelled *E. coli* marker dissociated ribosomes (Δ---Δ) were analyzed as described in the text. Intermediate S values between 30 and 50 are interpolated.

hundred fractions of 200 drops/tube were collected from each column and protein concentration was determined by measuring the A_{230} of the undiluted fractions as shown in Fig. 6.

Acrylamide gel electrophoresis at pH 4.6 (not shown) was performed on approximately 200 fractions in order to characterize the protein content of the several chromatographic peaks, and to identify the area where S 19 eluted.

The inset gel in Fig. 6 shows the region which contained S 19. Other proteins (S 4, S 7, S 11 and S 15) isolated in this region are also identified. This area of the elution pattern (Fractions 292 to 390) is called fraction L.

6. Phosphocellulose chromatography at pH 6.5. Previous experiments (15) had shown that S 15 and S 19 not only coelectrophorese at pH 4.6 but also coelute on Sephadex G 100 columns. Therefore it was necessary to find a method to separate S 15 and S 19.

Since the proteins in fraction L eluted when the salt concentration reached about 0.3 M at pH 8.00, it was decided to construct a 0.25 M to 0.45 M gradient and to lower the pH to 6.5 (31).

Fraction L was diluted 4-fold and concentrated on 4 small phosphocellulose concentrating columns as described above. The sample was then dialyzed until the salt concentration was 0.15 M LiCl and the pH adjusted to 6.5. Phospho-

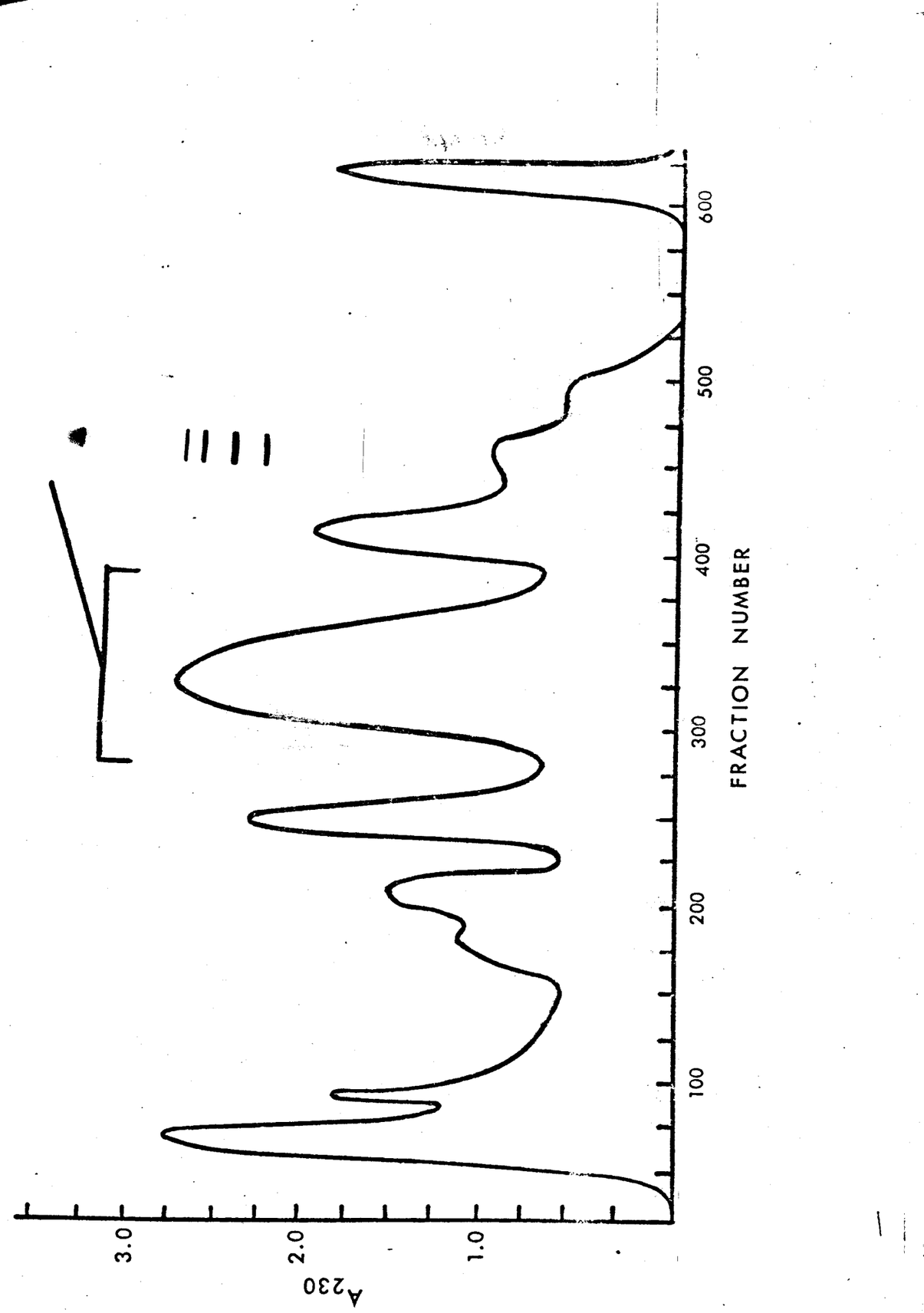

Fig. 6. Chromatogram of phosphocellulose pH 8 column chromatography. Methods are described in the text. Fraction L (285-395) is pooled. Inset pH 4.6 gel shows presence of 4 bands (S 4, S 7, S 11, S 15 and S 19).

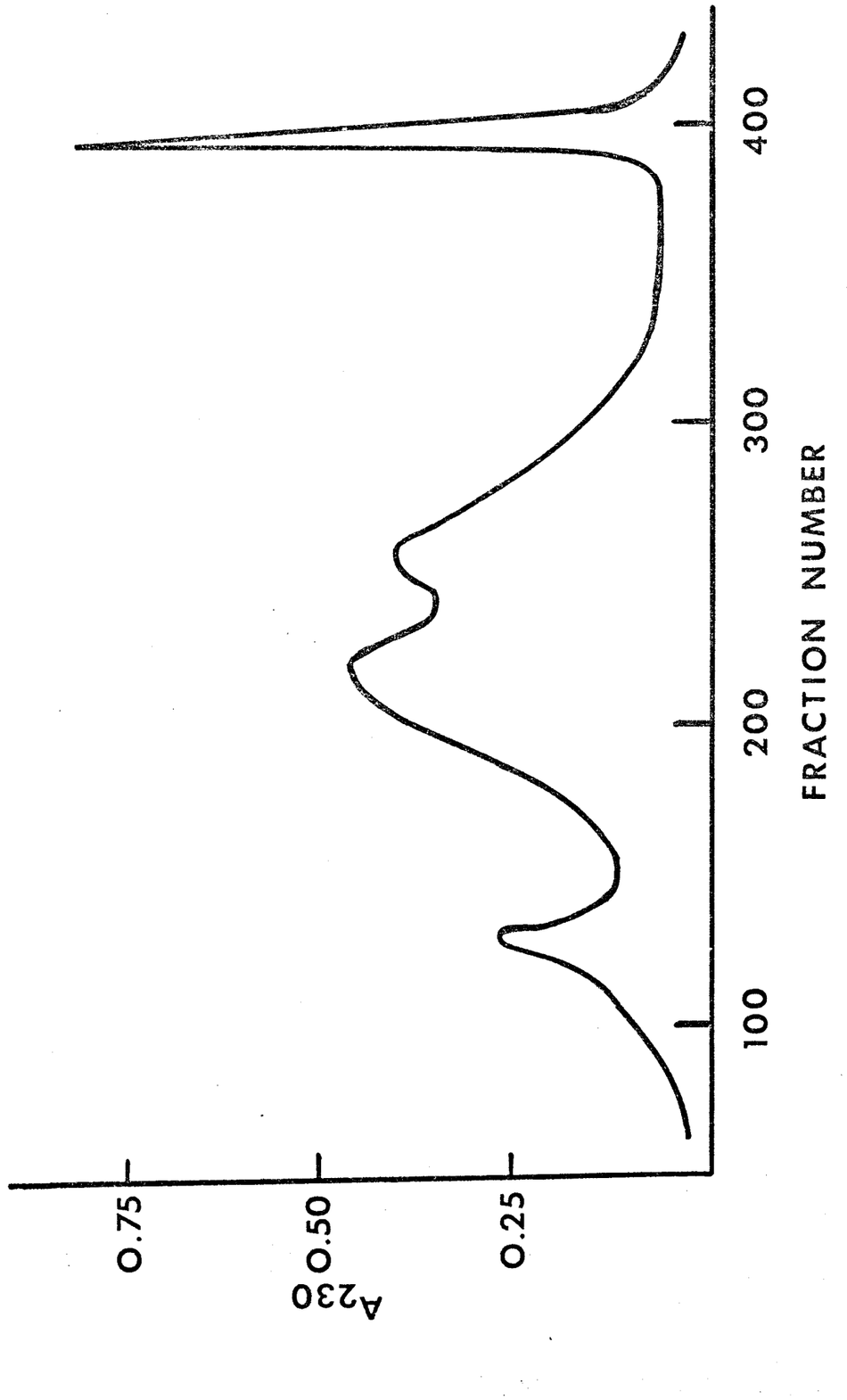

Fig. 7. Chromatogram of phosphocellulose pH 6.5 column chromatography. Fraction L is concentrated and chromatographed at pH 6.5 as described in the text. Fraction peak 392 contains only S 15.

cellulose chromatography at pH 6.5 was then performed as above and protein concentration was determined by A_{230}. The elution pattern shown in Fig. 7 shows four distinct peaks. Electrophoretic analysis at pH 4.6 of the peak fractions is shown in Fig.

It was shown in previous experiments that S 19 and S 15 coelectrophorese at pH 4.6. We see in Fig. 8 that gels from fraction 129, 225 and 255 all contain a band in the S 15-S 19 region. Fraction 392, which is the elution product of the 1 M LiCl wash, also shows a very strong band in this region. Since previous experiments had shown that S 19 migrates considerably faster than S 15 at pH 8.7 we ran gels of this pH in order to see if we had effected a separation of S 15 from S 19. The pH 8.7 gels depicted in Fig. 9 showed that S 15 has eluted in the 1 M wash and is only present in fraction 392. However, S 19 is spread through the entire chromatogram from fraction 112 to fraction 320. Also double diffusion experiments (not shown) with antiserum against *E. coli* S 19 (gift from L. Kahan) showed precipitation in all fractions tested except 392. Therefore we succeeded in separating S 15 from S 19.

Fractions 142 to 280 containing proteins S 4, S 7, S 11 and S 19 were then concentrated by phosphocellulose columns and ultrafiltration as described above.

7. Sephadex gel filtration. The concentrated fractions

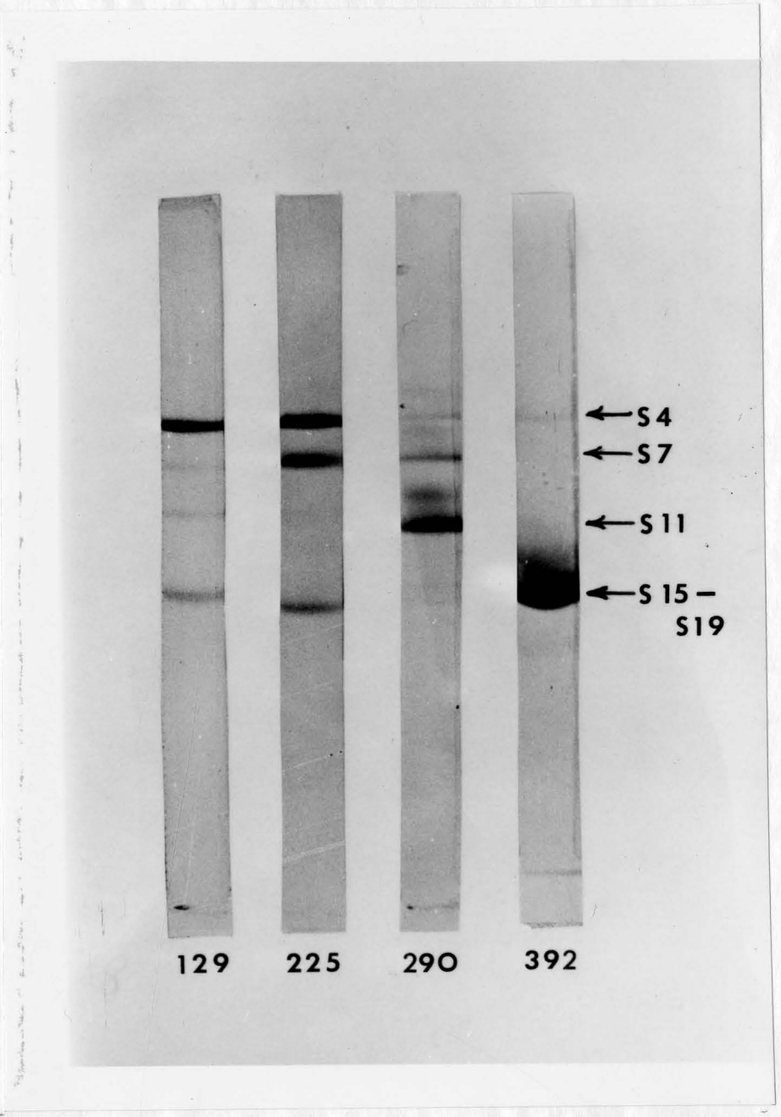

Fig. 8. pH 4.6 polyacrylamide gel electrophoretic analysis of pH 6.5 chromatogram. Gel from peak fraction 129 shows S 4, S 7, S 11, S 15-S 19. Fraction 225 gel shows S 4, S 7, S 15-S 19. Fraction 290 gel contains S 4, S 7, S 11. Fraction 392 gel contains a major band in the S 15-S 19 region. Fig. 9 shows this band to be S 15.

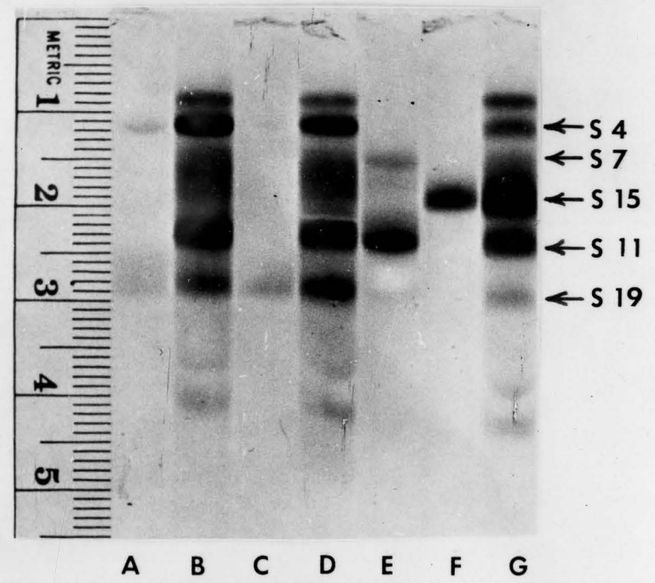

Fig. 9. pH 8.7 polyacrylamide gel electrophoretic analysis of pH 6.5 chromatogram. Gel A corresponds to fraction 129; Gel B is fraction 129 with total 30 S protein (TP30) marker added; Gel C is fraction 220; Gel D is fraction 220 plus TP30; Gel E is fraction 290; Gel F is fraction 392; and Gel G is fraction 392 plus TP30. Since S 15 and S 19 coelectrophorese at pH 4.6 this gel system is used to resolve these proteins into separate bands. Gel A contains S 4, S 7 and S 19. Gel C contains some S 4 and S 19. Gel E contains S 5, S 7 and some S 19. Gel F contains only S 15.

were separated by size on a Sephadex G 100 column in 0.15 M LiCl UMP pH 8.00. Ten ml fractions were collected and A_{230} was determined as shown in Fig. 10. The elution pattern shows 2 large incompletely separated peaks followed by one smaller peak. Electrophoretic analysis at pH 4.6 of fraction 122, peak fraction 130, shoulder fraction 150 is shown in Fig. 11. Protein S 4 elutes as the first peak, S 7 as the second and S 11 occupies the descending shoulder of the second peak. There is of course, extensive cross contamination in this region.

The elution pattern of the small Sephadex G 100 peak is expanded in Fig. 12. The pH 4.6 electrophoretic pattern of fractions 150, 154, 156, 158, 160, 162, 165, 170 and 180 are shown in the inset. In these gels we see that there is S 7 contamination visible until fraction 158 and S 11 contamination up to fraction 162. All subsequent gels show one homogeneous band. It was thus concluded that the S 19 fractions were free of S 5, S 7 and S 11 contamination after fraction 162. To ascertain that fractions were free of S 15, electrophoresis was performed at pH 8.7 Gels were overloaded with 100 times the normal amount of sample. It can be seen in Fig. 13 that at fraction 170 contamination is present at about the 5% level and is much less evident in fraction 180. It was therefore concluded that S 19 is essentially purified after fraction 170.

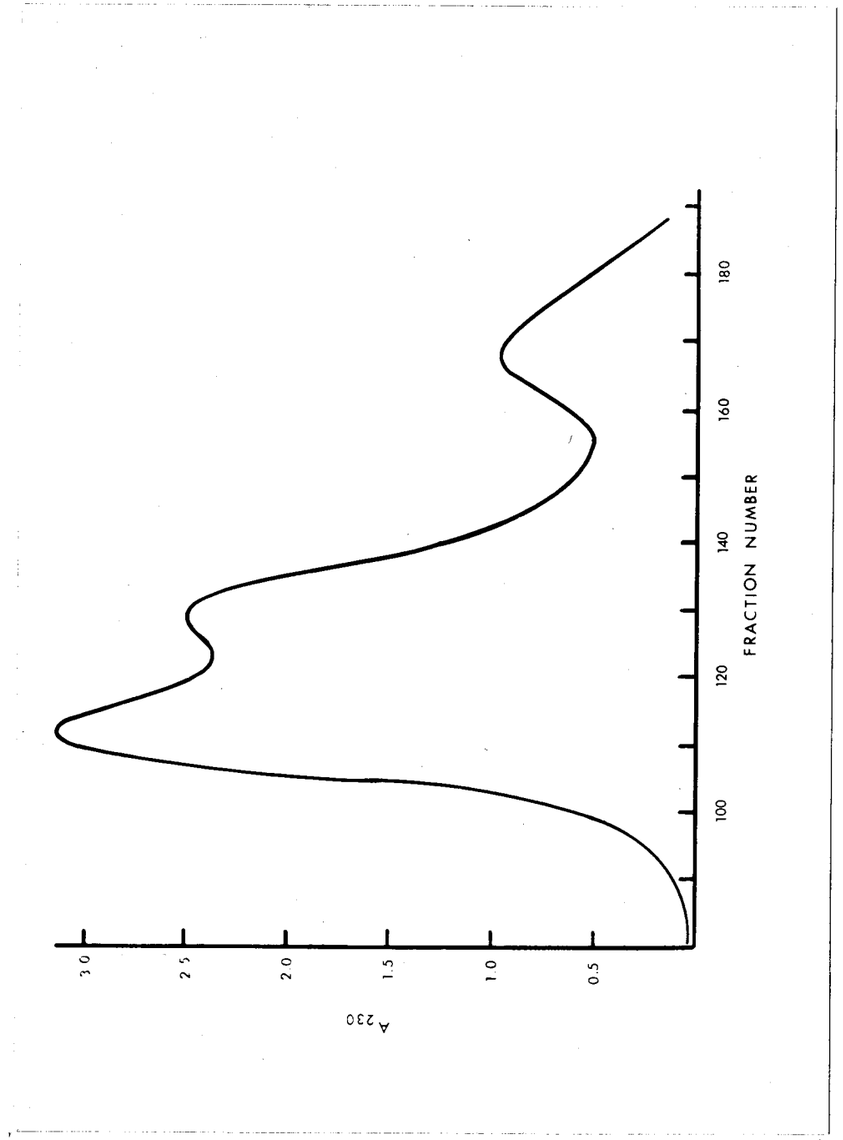

Fig. 10. Sephadex Gel Filtration. Peaks containing S 19 and no S 15 were pooled and subjected to Sephadex G-100 gel filtration as described in the text. The first two peaks contain primarily S 4 and S 7 as shown in Fig. 11. The last small peak contains primarily S 19 which is essentially pure after fraction 170. See Figs. 12 and 13.

Fig. 11. pH 4.6 polyacrylamide gel electrophoretic analysis of Sephadex gel filtration. Methods described above. We see peak 122 and 130 contain S 4 and S 7. Shoulder 150 contains S 4 (faint), S 7, S 11 and S 19.

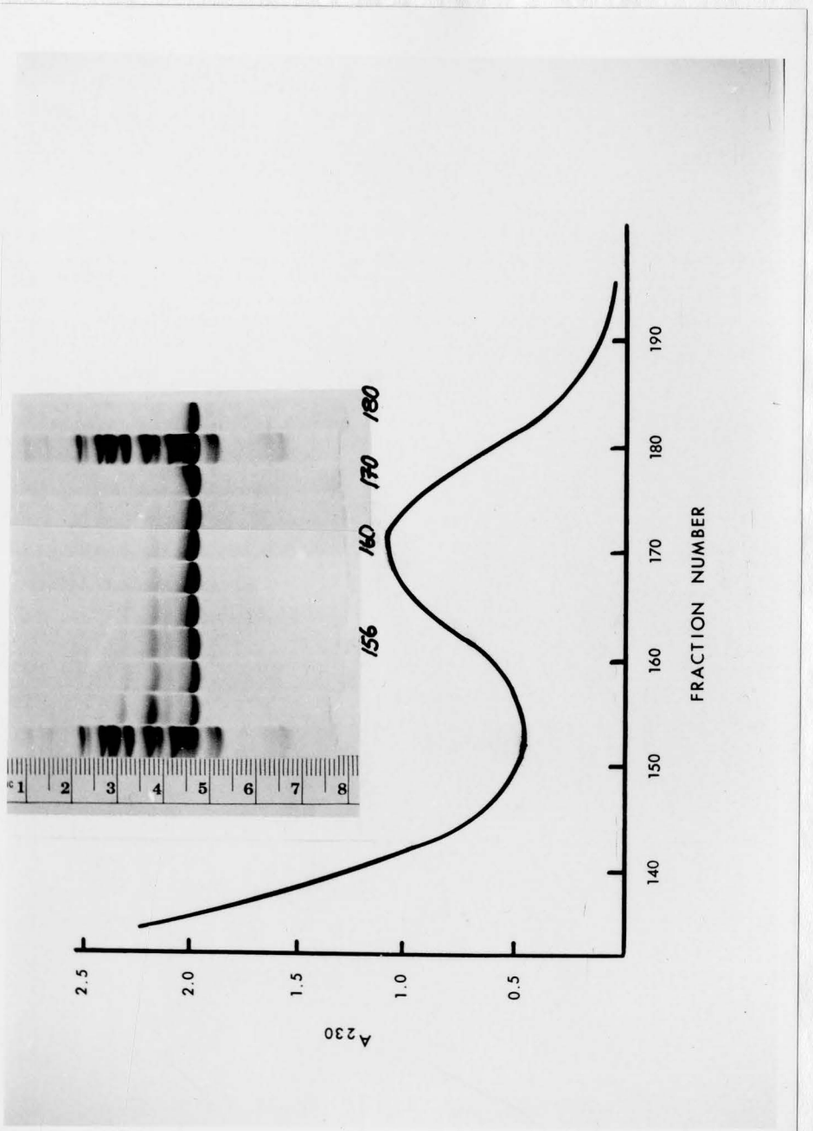

Fig. 12. Expanded section of Sephadex Gel Filtration with pH 4.6 electrophoretic analysis inset. In this figure the last peak of Fig. 11 is expanded. The inset gels show the separation of S 19 away from S 7 and S 11. After fraction 170, S 19 is essentially fractionated. Inset gels contain (from left) fractions: TP 30, 150, 154, 156, 158, 160, 165, 170, TP 30 and 180.

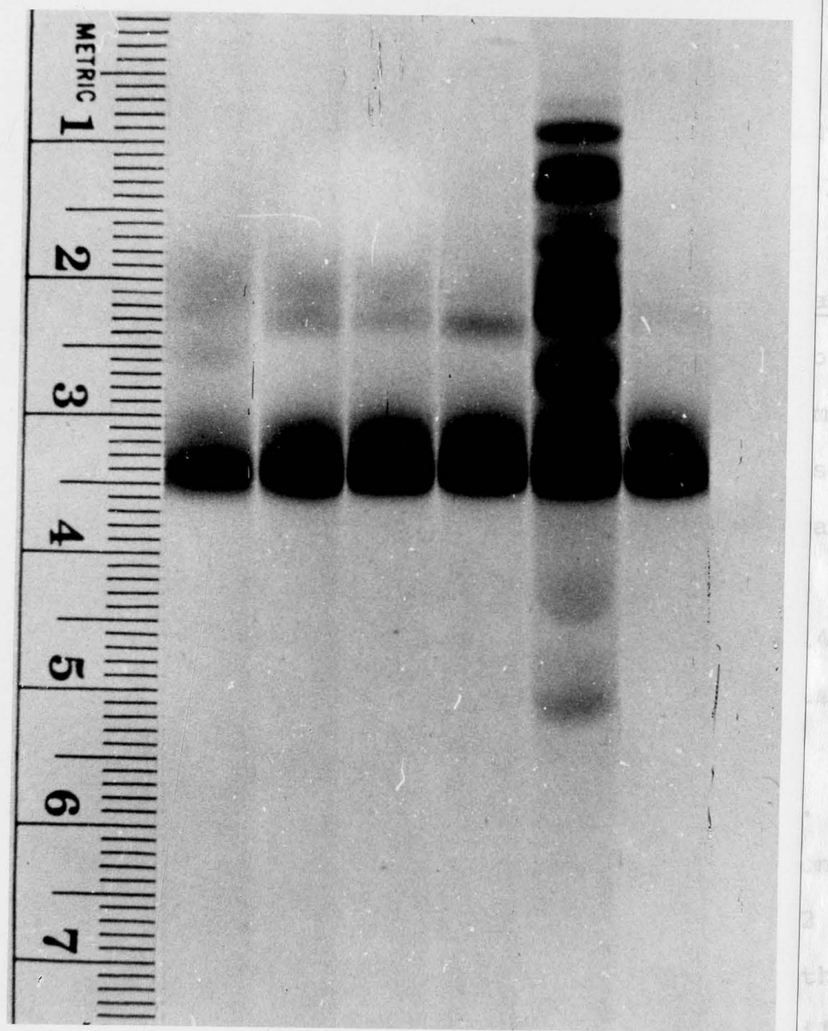

Fig. 13. pH 8.7 polyacrylamide gel electrophoretic analysis of last peak of Sephadex Gel Filtration. Gels (from left) contain protein from fractions: 156, 160, 165, 170, 170 + TP 30 and 180. Grossly overloaded gels show S 19 fractionation. Fractions 156, 160 and 165 show some (perhaps 10%) S 4, S 7 and S 11 contamination. Fraction 170 shows a small amount of S 15 contamination which is essentially gone by fraction 180. The order of protein migration is the same as in Fig. 9, in which pH 8.7 electrophoresis is used to resolve S 15 and S 19 which coelectrophorese at pH 4.6.

Two dimensional gel electrophoresis was done with 80 µg protein from fraction 171. The results, showing a strong S 19 spot and a very faint S 15 spot are compared with *Bacillus* 30 S proteins in Fig. 14.

Since the automated Beckman sequenator will tolerate up to 10% contamination, fractions 160-170 were used for this purpose. A portion of the remaining protein from 171-190 was used for SDS molecular weight determination and amino acid analysis. Unused protein samples were frozen and kept at -80 C.

8. Molecular weight determination by SDS - Acrylamide gels. Five µg each of S 19 and protein standards (carbonic anhydrase, trypsin inhibitor S, myoglobin and cytochrome C) were subjected to SDS acrylamide gel electrophoresis as described above. The mobility values express the average of triplicate determinations. The 3 determinations of S 19 ranged from 12,900 to 15,000 with an average of 14,000, as shown in Fig. 15. This molecular weight estimate is subject to an error of ± 10%.

9. N Terminal amino acid sequence determination. Fractions 160-170 from the Sephadex G 100 fractionation were pooled, concentrated and desalted on a Biogel P 2 column. The protein fractions from this column were then lyophilized, resuspended in distilled water and lyophilized again.

Three mg of the lyophilized protein were dissolved

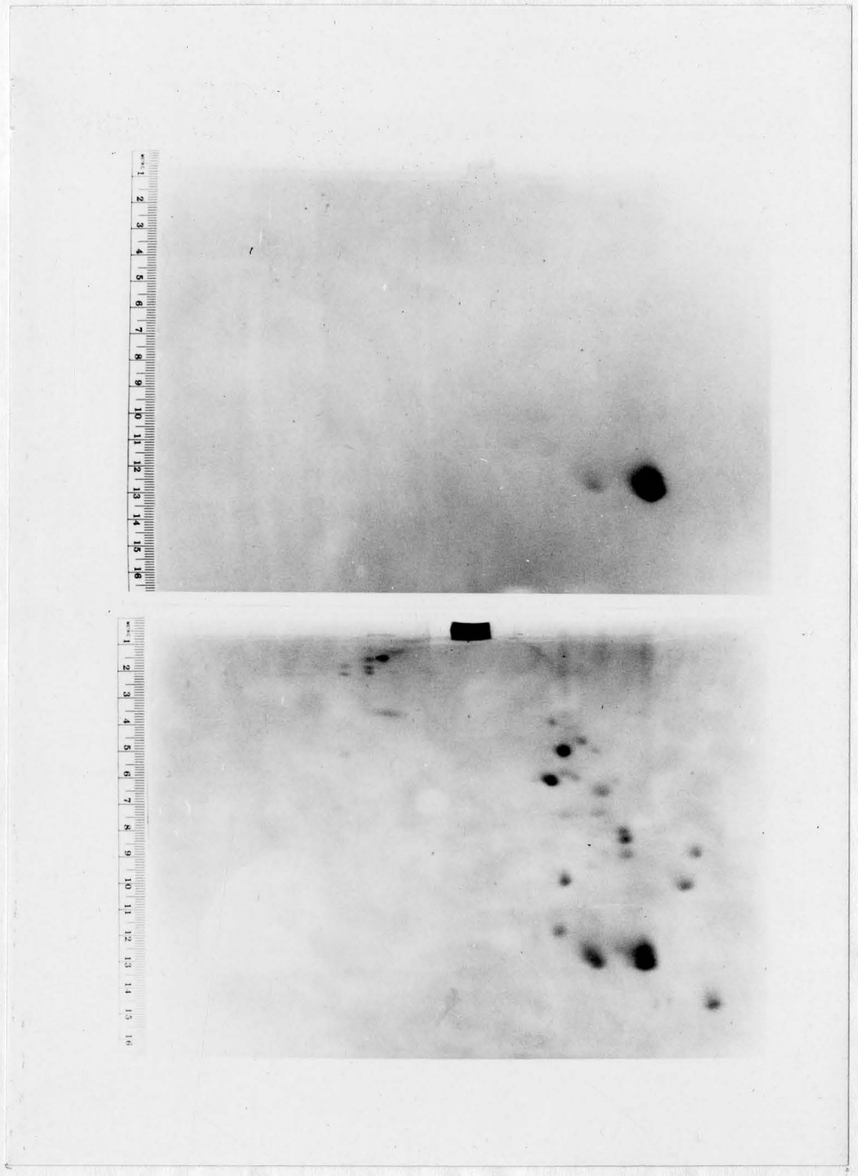

Fig. 14. 2-D polyacrylamide gel electrophoretic analysis of *Bacillus* S 19 and comparison with *Bacillus* TP 30. The top figure shows a 2-D gel of fraction 171 of the Sephadex gel filtration. We see two spots; the major S 19 spot and a small S 15 spot. The bottom gel shows fraction 171 run with a background of *Bacillus* TP 30. Methods are described in the text.

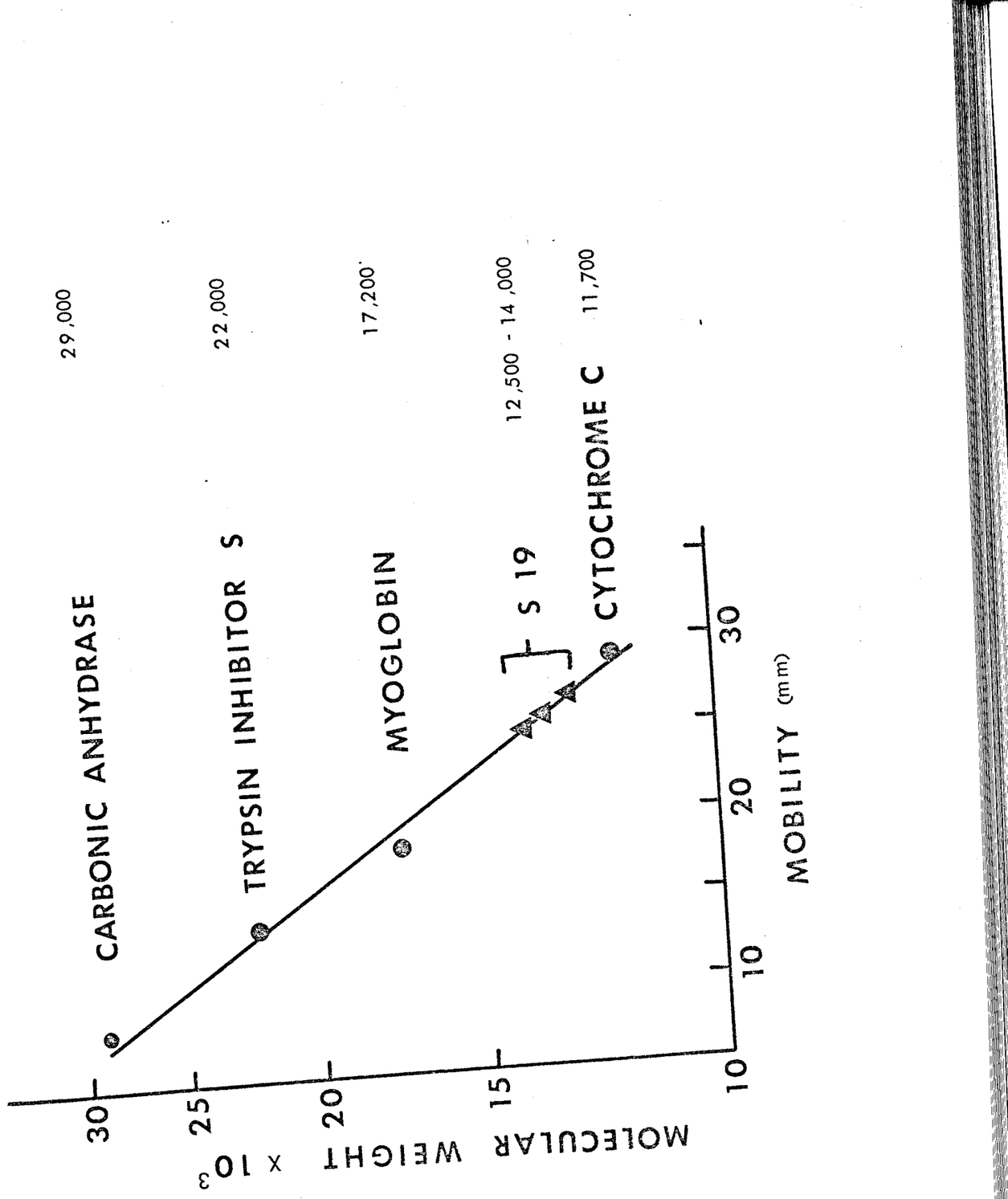

Fig. 15. Molecular weight determination of *Bacillus* S 19 by SDS gel electrophoresis. The mobility versus log molecular wt of triplicate determinations by SDS gel are shown. Materials and Methods are described in the text.

in formic acid and placed in the sequenator reaction cup. Ten µl butanethiol was added and the system sealed. The cup atmosphere was purged with nitrogen at 55 C followed by 30 min of "fine" vacuum. Automated sequencing was then performed and sequenator fractions converted as described above. Gas chromatography was performed on the PTH derivative of each residue and on the TMS derivative. On SP-400 the silylated (TMS) derivatives of alanine, glycine, valine, leucine, isoleucine, methionine, phenylalanine, proline and tyrosine chromatograph very well and are easily quantitated. PTH-tryptophan, serine and threonine are labile and PTH lysine, asparagine and glutamine chromatograph poorly. By silylation it is possible to differentiate between isoleucine and leucine. Table 2 shows the results of gas chromatographic analysis of the first 30 N-terminal residues of *Bacillus* S 19. The second and third columns show the results of gas chromatography with and without silylation and column one shows the final deduced residue. Residues not assignable by gas chromatography will be subjected to amino acid analysis. The repetitive yield computed from the yield of residue 1 (glycine) and residue 7 (glycine) was 96.5%.

10. Amino acid analysis. Four nM S 19 as determined by the Folin-Lowry method were dissolved in 1 ml UMP and aminoethylated. The protein was then dialyzed extensively against 0.6% acetic acid and lyophilized in an 18 x 150 mm

TABLE 2: AMINO ACID SEQUENCE DETERMINATION OF *BACILLUS* S 19

Step No.	Deduced Res.	G.C. SP-400	TMS	Step No.	Deduced Res.	G.C. SP-400	TMS
1	Gly	Gly	Gly	16	Lys	Lys	Lys
2		---	---	17	Lys	Lys	Lys
3	Ser	Ser	Ser	18	Ile	Ile/Leu	Ile
4	Leu	Leu/Ile	Leu	19	Lys	Lys	Lys
5	Lys	Lys	Lys	20	Lys	Lys	Lys
6	Lys	Lys	Lys	21	Leu	Ile/Leu	Leu
7	Gly	Gly	Gly	22		---	---
8	Pro	Pro	Pro	23		---	---
9	Phe	Phe	Phe	24		---	---
10	Ser	Ser	Ser	25	Gly	Gly	Gly
11		---	---	26		---	---
12	Gln	---	Gln	27	Lys	Lys	Lys
13		---	Lys	28		---	---
14	Leu	Ile/Leu	Leu	29	Val	Val	Val
15	Met	Met	Met	30	Ile	Ile/Leu	Ile

borosilicate glass test tube. Two ml 6 N HCl was added and the samples hydrolized as described above for 20 hr at 110 C. The amino acid composition was then determined on a JEOL AH6 using the two column system. With this system greater than 90% resolution of all amino acid peaks was obtained and 0.1 nm of amino acid gave a detectable peak. Peak areas were automatically and hand integrated. Compositions were calculated using color factors for standard amino acid mixtures (Pierce) supplemented with S-2-aminoethylcysteine (Sigma) and cysteic acid (Sigma). Table 3 shows the amino acid composition of S 19 calculated as mole per cent minus tryptophan and ammonia.

Minimum molecular weight was also determined from the amino acid composition, choosing the amino acid which appeared least frequently. Quantitatively, Mmin = cysteine corrected molecular weight X 100/percentage of cysteine. The corrected molecular weight of cysteine in the peptide linked form is 123-18 or 105. Therefore the minimum molecular weight is 15,000.

TABLE 3: *BACILLUS* S19 (P13)

AMINO ACID COMPOSITION

(minus NH_3 and Trp)

EXPRESSED AS MOLE %

Lys	13.5	Ser	3.6	Val	6.2
His	5.2	Glu	9.6	Met	2.1
Arg	8.4	Pro	3.7	Ile	5.1
Asp	7.1	Gly	10.5	Leu	5.0
Thr	8.1	Ala	4.2	Tyr	2.4
Phe	5.3	Cys	0.7		

DISCUSSION

Since S 19 has been implicated to have a major role in protein synthesis a brief review is presented. Current views of ribosome function are based on the "two-site" model proposed by Watson (50). Peptide bond formation is described as a cyclic process in which the 30 S subunit provides a binding site for mRNA and a second binding site (A site) for accepting aminoacyl tRNA prior to the formation of the peptide bond. The 50 S subunit provides a binding site (P site) for peptidyl tRNA. After the formation of a peptide bond the system is altered by 1) a different tRNA attached to the growing polypeptide chain in the P site and 2) the advance of the mRNA so that a new codon can direct the selection of the next aminoacyl tRNA for the A site. The several consequences of this model and the complexities of the translation process itself emphasize the distinction between possession of the flow diagram of genetic information transfer and the understanding of the mechanism of this process. Issues raised by the Watson model have separated studies of the structure and function of the ribosome and protein synthesis into 3 major areas: polypeptide chain initiation, chain elongation and chain termination.

Nathans and Lippmann (30) first demonstrated that supernatant proteins are required for polypeptide synthesis and subsequently, several proteins were characterized which

are transiently associated with the ribosome during the different stages of protein synthesis. Three protein factors have been implicated in the initiation phase (IF-1, 2, 3). IF-3 seems to be required to bind mRNA to the 30 S subunit. IF-2 stabilizes the IF-3·mRNA·30 S complex and more specifically guides a specific tRNA (fMet $tRNA_f$) to this complex. Upon the binding of GTP and IF-1 to this conglomerate, IF-3 is released and the 30 S·IF-2·GTP·fMet $tRNA_f$·IF-1 "initiation complex" is formed. The 50 S subunit is then bound to this complex, IF-1 is released, and GTP hydrolysis occurs releasing inorganic phosphate. GTP hydrolysis is implicated in the translocation of the fMet tRNA from the A site to the P site and in the release of IF-2 from the complex. When the fMet $tRNA_f$ is in position on the ribosome, chain elongation mediated by the appropriate factors can begin.

Two supernatant proteins (T_s and T_u) and GTP contribute to the chain elongation process. GTP binds with T_u and aminoacyl tRNA to form the aminoacyl tRNA·GTP·T_u complex. T_s accelerates this complex formation. This complex then binds to the A site of the ribosome. Peptide bond formation occurs by a nucleophilic reaction between the amino group of the aminoacyl tRNA and the carboxylic ester of the fMet tRNA. This reaction is catalyzed by the enzyme peptidyl transferase (which is one of the 50 S subunit proteins) and

is dependent on GTP hydrolysis. Subsequently T_u·GDP and inorganic phosphate are released. After the peptide bond has been formed, the peptidyl tRNA occupies the A site and must be translocated to the P site before the next aminoacyl tRNA can enter in the A site. Simultaneously the deacylated tRNA is expelled from the P site and the mRNA moves along three nucleotides with respect to the ribosome in such a way that the next nucleotide triplet codon is in the correct position at the A site. Factor EF-G causes the GTP dependent translocation of peptidyl tRNA and mRNA.

Genetic experiments as well as those performed *in vitro* with synthetic mRNA molecules indicate that the codons UAA, UAG and UGA signal termination of protein synthesis. Three protein factors have been implicated in the termination step. When translocation places one of the nonsense codons in the A site the ribosome does not bind an aminoacyl tRNA·EF-T_u·GTP complex. Instead it binds a protein (R_1 or R_2) which activates peptidyl transferase and subsequently hydrolyzes the bond joining the polypeptide to the tRNA in the P site.

Protein synthesis has been most recently reviewed by Lucas-Lenard (27). The purpose of this brief review is to show the current state of the art since the introduction of the two site model. The identification of the functional roles played by the supernatant factors has cast a rather

different light on protein synthesis: the ribosome is no longer viewed as a self contained apparatus for the functional apposition of tRNA and mRNA molecules. We now see that at least nine protein factors shuttle back and forth on the ribosome, and that the interactions between the ribosome, mRNA and tRNA are guided by some of these factors, while other factors guide the ribosome-mediated processes of peptide bond formation, translocation and mRNA movement. Thus the protein synthetic apparatus is much more dynamic than it was originally thought to be.

So far, we have discussed the functional aspects of protein synthesis apart from the structure of the ribosome since just as the functional analysis of the various supernatant factors has depended on their purification so too the functional analysis of the individual ribosomal constituents has depended upon their isolation in "pure" form. At this time, the functional properties of only a handful of ribosomal proteins (aside from assembly functions described above) has been demonstrated (23). Randall-Hazelbauer (36) has shown that proteins S 2, S 3 and S 14 stimulate the tRNA binding capacity of the ribosome thus assigning them to the A site. S 11 and S 21 (5, 23) have been shown to contribute to mRNA and tRNA binding, and S 1 has been shown to stabilize the initiation complex. Considerable effort is now being made to identify the functions of the individual proteins

and to determine the relationships between their amino acid sequence and function. Since as has so often been the case in protein chemistry information concerning active sites and other important conserved regions has come from the comparison and contrast of two different proteins having the same function we have partially characterized *Bacillus* S 19 in order to compare it with *Escherichia* S 19.

We fractionated *Bacillus* S 19 into a nearly homogeneous state as described above. It is a basic protein with a pI greater than 12 and migrates toward the cathode in both pH 4.6 and pH 8.7 acrylamide gels. It is present in an oxidized and reduced form in these gels but the oxidized form can be eliminated by the addition of 2-mercaptoethanol. The molecular weight of *Bacillus* S 19 has been estimated by two methods. SDS-gels give an apparent molecular weight of 14,000 $\pm$ 10% and calculation of minimum molecular weight by amino acid analysis yields a molecular weight of 15,000 daltons. Since there is reasonably good agreement by these two methods we conclude that *Bacillus* S 19 is a small polypeptide having a molecular weight of about 15,000 daltons and contains between 108 and 123 amino acid residues.

The amino acid analysis of S 19 has been done on duplicate acid hydrolysates of native S 19 and aminoethylated S 19; the mole percent of each amino acid has been calculated minus ammonia and tryptophan, ignoring the possible amide

forms of glutamate and aspartate. Mole percents total 100.7 Examination of Table 3 shows that S 19 like most other 30 S proteins is very basic and rich in lysine and arginine. The basic hydrophilic amino acids account for 27% of the residues; the acidic hydrophils and their amides (if any) comprise 19% of the residues. Neutral and hydrophobic amino acids make up respectively 27% and 26% of the residues.

Table 2 shows the gas chromatographic assignment of residues extracted from *Bacillus* S 19 by the Beckman 890 sequenator. By analysis of the phenylthiohydantoin and trimethylsilyl derivatives, positive identification of the first 30 residues except: 2, 11, 13, 22, 23, 24, 26 and 28 has been made. By subsequent hydrolysis of sequenator samples and amino acid analysis we have been able to identify residues: 2 = Arg; 11 = Asx; 13 = His; 22 = Asx; 23 = Glx and 24 = α amino butyric acid = Thr. Thus we have been able to positively identify 28 of the first 30 residues.

We have examined the possibility of α-helix formation by plotting the linear amino acid sequence into helical "wheels" according to Schiffer and Edmundson (38). Since it is permissible for proline to be part of the first or last turn of an α-helix we began plotting our sequence with residue 8 (proline). Adjacent residues: 8 (Pro); 15 (Met); 18 (Ile); 25 (Gly); 14 (Leu); 21 (Leu) all appear in a

hydrophobic arc. It is even more intriguing however that residues: 19 (Lys); 12 (Gln); 23 (Glx); 16 (Lys); 20 (Lys); and 13 (His) form an almost continuous 180° arc in the helix. It is immediately obvious that this sort of helix would have a definite spatial orientation with the basic and hydrophilic portion being exposed to solvent-protein interactions while the opposite hydrophobic arc is involved in protein "interior" interactions. Upon further analysis however we have decided that there are an insufficient number of hydrophobic residues to form the "stabilization arc" necessary for maintainance of an α-helix from residues 8 to 29. It is possible however that a smaller portion of this region (12 to 21) may be in an α helical form. These models are of course highly speculative and ORD and x-ray data will be needed for these determinations.

Next we will consider the data concerning protein S 19 from *Escherichia*. (The amino acid composition data provided by Dr. Lawrence Kahan, and the unpublished N-terminal sequence data provided by Ken-Ichi Higo as personal communications are gratefully acknowledged.) The molecular weight of *Escherichia* S 19 has been analyzed by SDS gels (46) and by analytical ultracentrifugation (9) to be about 15,000 daltons. Since it has been noted that the *Bacillus* and *Escherichia* S 19's are interchangeble in reconstitution and will cross-react immunologically, it is perhaps not

surprising that they should have similar molecular weights.

Next we shall compare the amino acid composition of *Bacillus* and *Escherichia* S 19. The *Escherichia* composition data differs slightly from laboratory to laboratory and there seems to be some question as to whether it contains any cysteine. The preparations of Dr. Nomura's group do not. Table 4 shows the amino acid compositions of *Bacillus* and *Escherichia* S 19. We see that there is no difference in terms of the basic hydrophilic residues but a 3% difference is found in glutamic acid content. It is possible however that some of these extra acidic residues in *Bacillus* are present as the amide form: glutamine. Overall, there is only about a 10% difference in the residues.

Finally we come to the comparison of the N-terminal amino acid sequences of *Bacillus* and *Escherichia* S 19, shown in Table 5. It is immediately obvious that *Escherichia* S 19 begins with a very unusual residue - proline. It was originally feared that *Escherichia* S 19 was degraded somehow during preparation and that a fragment beginning with proline was being sequenced, however, when *Bacillus* S 19 was found to contain a homologous N-terminal region, this fear was relieved. *Bacillus* S 19 does however begin with a more "normal" glycine residue. Eight of the first 9 amino acids are identical in these two protein, and it is possible that this region is of such functional significance as to be

TABLE 4: COMPARISON OF AMINO ACID COMPOSITION:

BACILLUS AND *ESCHERICHIA* S 19

A.A.	B.	E.	%d.	A.A.	B.	E.	%d.	A.A.	B.	E.	%d.
Lys	13.5	13.5	0	Glu	9.6	6.06	3	Ile	5.1	4.46	0
His	5.2	5.16	0	Pro	3.7	6.17	2	Leu	5.0	7.9	3
Arg	8.4	8.34	0	Gly	10.5	8.20	2	Tyr	2.4	1.08	1
Asp	7.1	6.99	0	Ala	4.2	7.05	3	Phe	5.3	4.63	1
Thr	8.1	6.22	2	Val	6.2	7.64	1	Cys	0.7	0	1
Ser	3.6	4.23	1	Met	2.1	2.19	0				

TABLE 5: COMPARISON OF N-TERMINAL AMINO ACID SEQUENCES:

BACILLUS AND *ESCHERICHIA* S 19

	1	2	3	4	5	6	7	8	9	10
	CCZ	A CGZ	UCZ	U CUZ	UU AAY	AAY	GGZ	CCZ	UUY	AUZ*
E.C.	Pro	Arg	Ser	Leu	Phe Lys	Lys	Gly	Pro	Phe	Ile
B.S.	Gly	Arg	Ser	Leu	Lys	Lys	Gly	Pro	Phe	Ser
	GGZ	CGZ A	UCZ	U CUZ	AAY	AAY	GGZ	CCZ	UUY	AGY UCZ
	11	12	13	14	15	16	17	18	19	20
	GAX	CUZ	CAX	U CUZ	U CUZ	AAY	AAY	GUZ	GAY	AAY
E.C.	Asp	Leu	His	Leu	Leu	Lys	Lys	Val	Glu	Lys
B.S.	Asp	Glx	His	Leu	Met	Lys	Lys	Ileu Lys	Lys	Lys
	GAX	GAY CAG	CAX	U CUZ	AUG	AAY	AAY	CUZ	AAY	AAC
	21	22	23	24	25	26	27	28	29	30
	GCZ	GUZ	C Y GAG	AGY UCZ	GGZ	GAX	AAY	AAY	CCZ	U CUZ
E.C.	Ala	Val	Glx Glu Tlc	Ser	Gly	Asp	Lys	Lys	Pro	Leu
B.S.	Ileu Leu	Asx	Glx	Thr	Gly		Lys		Val	Ile
	A CUZ*	GAX	GAG CAY	ACZ	GGZ		AAY		GUZ	AUZ*

absolutely conserved. After residue 9, the homology is less obvious but most amino acid changes are conservative: 15 (Leu to Met), 18 (Val to Ile), 21 (Ala to Leu), and 24 (Ser to Thr). There are several non-conserved changes which may not be too disruptive: 10 (Ile to Ser), 12 (Leu to Gln), 22 (Val to Asx), and 29 (Pro to Val). In the first 30 residues there is only one totally non-conservative change: from glutamic acid to lysine in residue no. 19. This may or may not be disruptive since the requirement of that region may be for a polar residue irrespective of change. Overall we see an identical region from residues 2 to 9, a region of change from 10 to 12 and a new region of inexact homology from 13 to 30 with major changes at 19 and 22.

Table 5 also shows the mRNA codon assignments for the first 30 amino acids. Because of third base wobble this base is often not assignable and the mutations occurring there are not detectable by amino acid replacement. We see a G to U transversion in base 2 of codon number 10; A to U transversion in codon 12; A to U or C in codon 18; A to G transition in codon 19; A to U transversion in codon 24. Codons 1, 21 and 22 require 2 codon mutations to account for the amino acid replacements.

While it is clear that *E. coli* and *Bacillus* S 19 are related functionally, it is apparent now that they also

possess a degree of evolutionary homology. We now have glimpsed this evolutionary and functional relationship by comparison of sequence and amino acid content, but the more definitive statement of homology must await the comparisons of the tryptic "fingerprints" of these proteins.

Lastly, I would briefly relate new information concerning the *in vivo* and *in vitro* functions of S 19. As we have stated earlier several proteins have been implicated in the A site of the 30 S. A recent series of experiments by A, Bollen and R. Traut (personal communication) have indicated that S 19 is the major contributing protein of the A site, and is involved in binding of IF-2 and fMet tRNA. They have found that the presence of bound fMet tRNA protects proteins S 3, S 6, S 18, S 19 and S 21 from tryptic digestion. Further experiments have shown that the binding of fMet tRNA is inhibited, 80% by anti-S 19 antibody and 50% by anti-S 21 antibody. Binding of IF-2 to the 30 S also protects S 19 from anti-S 19 antibody. Finally they have subjected the "initiation complex" to the bifunctional cross-linking reagent suberimidate. They have been able thus to covalently link radioactive IF-2 to S 1, S 9, S 10, S 13, S 14 and S 19. It has been found that most of the radioactive IF-2 is bound to S 19. It is our belief that our partial characterization of S 19 and future elucidation of the physical

and functional aspects of this protein will lead to a greater understanding of the process of initiation and of protein synthesis itself.

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APPROVAL SHEET

The thesis submitted by Artemios Vassos has been read and approved by the following Committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

Dec. 21, 1973
Date

Tadayo Hashimoto
Chairman's Signature